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ORIGIN AND FUNCTION OF THE PROTOPLASMIC CONSTITUENTS IN PELOMYXA CAROLINENSIS

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Introduction

The protoplasmic constituents of several species of *Pelomyxa* have been described by Greeff (1874), Gould (1894), Wilson (1900) and Wilber (1942) and some suggestions have been made concerning their origin and their function but none of these suggestions are supported by experimental evidence. It, therefore, seems desirable to study the origin and function of these structures experimentally.

Relative Specific Gravity of the Protoplasmic Constituents

Open bottom hematocrit tubes were made of pyrex glass. The smaller opening was closed with a rubber band and the tubes were half filled with a heavy solution of gum-arabic in culture fluid. Then about 1 cc. of culture fluid, containing 5 or 10 pelomyxae, was carefully added and the solutions at the interface stirred with a glass rod and mixed so as to produce in this region a gradient of density and viscosity. The tubes were then put into an electric centrifuge and rotated, after which the rubber bands were quickly removed and the contents poured into partially frozen culture fluid, so as to prevent recovery of the pelomyxae. Some of these centrifuged, chilled pelomyxae were put into each of the following: one per cent osmic acid in culture fluid, ninety-five per cent alcohol, Champy's fluid, Regaud bichromate-formol mixture, and Bouin's fluid. These preparations were then studied under high and low magnifications. The results obtained are presented in Figure 1 and the following paragraphs.

Figure 1 shows that the pelomyxae during centrifugation were much elongated, that the refractive bodies (Wilber, 1942) aggregated at one end and the fat globules and contractile vacuoles at the other and that the food-vacuoles, the nuclei, the crystals, the beta granules, and the clear hyaloplasm respectively in fairly definite layers between; but it does not show which end is heavier.

Numerous specimens were consequently withdrawn from the tubes immediately after centrifugation, by means of an ice-cold capillary pipette of such small bore

[1] The writer wishes to express his sincere gratitude to Dr. S. O. Mast, who suggested the problem and under whose direction the work was done, for constant encouragement and invaluable aid in preparing the manuscript.

that they could not turn end for end. The capillary was then immersed in nujol, to eliminate distortion caused by the curved side walls, and examined under the compound microscope. Invariably, the refractive bodies were at the centrifugal and the fat globules at the centripetal end. The refractive bodies, consequently,

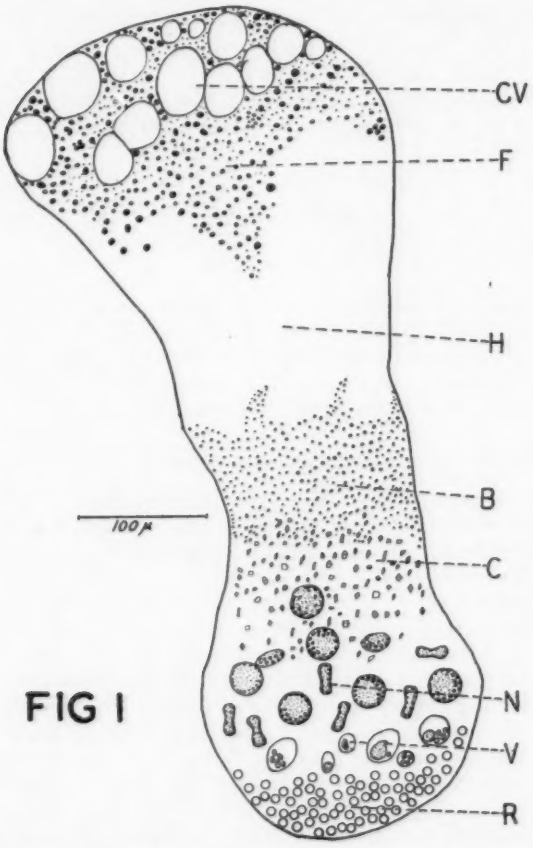


FIGURE 1. Camera sketch of a centrifuged *Pelomyxa* showing stratification of the protoplasmic constituents. R, refractive bodies (at centrifugal end); V, food-vacuoles; N, nuclei; C, crystals; B, beta granules (mitochondria); H, layer containing only hyaloplasm; F, fat; Cv, contractile vacuoles.

are the heaviest and the fat globules and contractile vacuoles the lightest constituents of *Pelomyxae carolinensis*. Mast and Doyle (1935) obtained similar results in observations on *Amoeba proteus*. However the substance in the nuclei did not stratify as in *Amoeba proteus*, indicating that either all the nuclear material has the

same specific gravity or that the viscosity of it is so high that the granules do not stratify under the centrifugation used.

The crystals were free in the cytoplasm, and not in vacuoles as occurs very commonly in uncentrifuged specimens and in centrifuged amoebae (Mast and Doyle), and many of them were broken probably by striking one another during centrifugation. Apparently, at the centrifugation used the crystals were thrown out of the vacuoles and aggregated in a different layer.

Recovery of Centrifuged Pelomyxae

Pelomyxae were centrifuged as described above and put on slides and examined under high magnification while the temperature slowly increased to that of the room. As the temperature increased, the plasmasol (Wilber, 1942) began to flow toward the centripetal end in all but a few specimens in which very small, stubby pseudopods first formed at the centrifugal end but these were soon withdrawn and the plasmasol began to flow in the opposite direction. The refractive bodies were the first protoplasmic constituents to enter the flowing plasmasol. They were carried toward the centripetal end and became distributed in the lower layer of the plasmasol. After the refractive bodies were all in the stream the food-vacuoles were carried forward and came to lie above the refractive bodies; then the crystals and beta granules were carried forward. In this manner the protoplasmic constituents were redistributed through the cell. The pelomyxae remained in the monopodal form during and for some time after the redistribution. The entire process required about 2 minutes.

Function of Nuclei

Pelomyxae were mounted on a slide without a cover-slip, examined under the high power of the binocular dissecting microscope, and left undisturbed until they had attached and had extended one or more pseudopods. Then, with the aid of a fine glass needle, pseudopods which contained refractive bodies, crystals, beta granules, and contractile vacuoles, but only a few or no nuclei, were cut off and mounted on a slide under a cover-slip supported by a ring of vaseline, some with numerous chilomonads, others with no food. The slides were examined under the low and high power of the compound microscope to ascertain the effect of removing various numbers of nuclei, on locomotion, feeding, digestion, and longevity.

Locomotion: After the operation, most of the fragments remained quiet for 30 to 120 minutes. Then all except those with no nuclei became active and soon moved precisely like normal specimens in all respects, and sudden increase in the intensity of light caused them to stop moving, just as it does normal specimens. Moreover, the method of formation of pseudopods was the same as in normal pelomyxae except that those with less than 9 or 10 nuclei invariably assumed the monopodal form which is not characteristic of uninjured pelomyxae.

All the anucleate fragments were sluggish. Coarsely granular material was aggregated in the center leaving a peripheral layer with no cytoplasmic constituents except beta granules; but usually there was no clear hyaline layer, although some few did have a distinct hyaline cap. The process of locomotion was greatly modified. In fact, most of the fragments did not move at all unless stimulated strongly.

These results are like those obtained by Verworn (1909), Gruber (1912), Stolć (1910) and Willis (1916) in observations on *Amoeba*.

Feeding and Digestion: Numerous fragments obtained as described above were examined carefully. All nucleate fragments ingested food and the process was the same as in uninjured pelomyxae. Fragments with 1, 3, 6, and 10 nuclei respectively were stained with neutral red and the process of digestion carefully observed. No difference from the normal was evident. Refractive bodies were formed as in normal specimens. The increase in number observed in 36 hours after feeding is given in Table I. This table indicates that the number of refractive bodies formed varied inversely with the number of nuclei.

TABLE I

Table showing the relation between the number of nuclei and the increase in number of refractive bodies in nucleate fragments of *Pelomyxa carolinensis*.

Number of nuclei in each fragment	Number of refractive bodies		Increase in per cent	Average
	Before feeding	36 hours later		
1	10	16	60	63
1	3	5	66+	
3	9	13	44+	45
3	4	5	25	
3	6	10	66+	
6	12	17	41+	39
6	8	11	37+	
10	31	30	0	0

Ten anucleate fragments were studied. Seven of these did not ingest anything; three ingested one chilomonad each. One of these was observed during the process of ingestion. It formed a food-cup which was apparently normal. The ingested chilomonads died in the food-vacuoles but were not digested. One of the fragments was sketched 24 hours after it had ingested a chilomonad (Fig. 2F). The chilomonad was then dead but still intact and there was no evidence of digestion even after 72 hours. A partially digested chilomonad was in a food-vacuole in this fragment at the time of operation; digestion proceeded no further (Fig. 2F′).

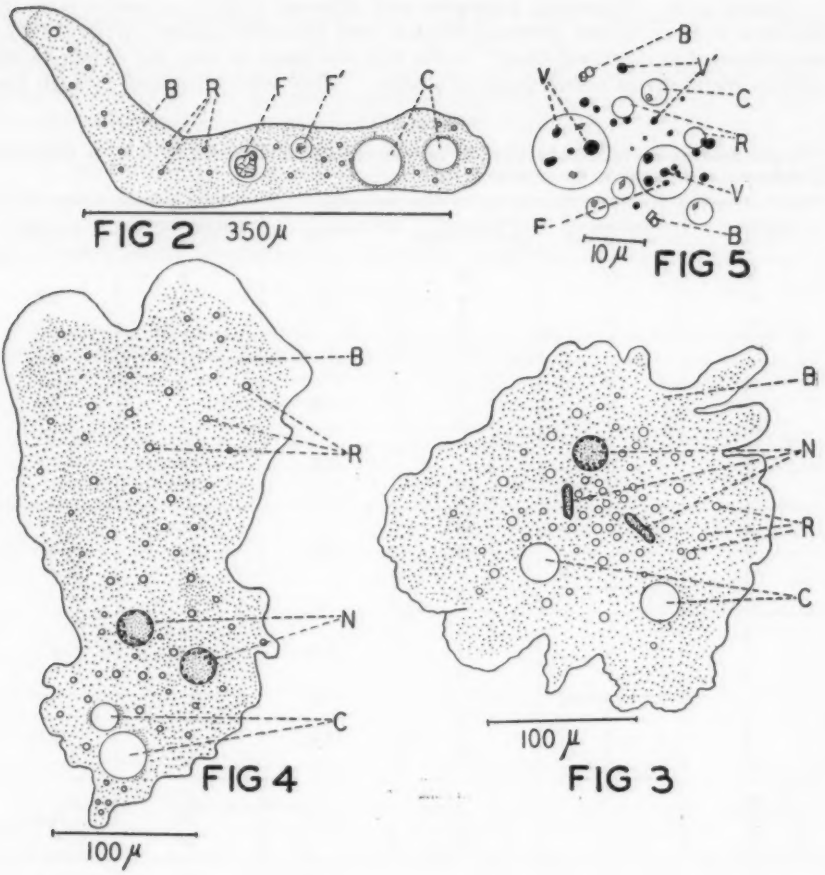
These results indicate that anucleate fragments of *Pelomyxa* sometimes ingest food and kill it but that they do not digest it, or food which was ingested before the operation. The results agree in part with those of Stolć (1910) who maintains that anucleate amoebae ingest food normally for several days. They are not in accord with the views of Hofer (1890), Gruber (1912), Willis (1916), Lynch (1919), and Becker (1926) who contend that anucleate amoebae do not ingest food. Becker, however, maintains that in anucleate fragments of *Amoeba dubia* "Food once ingested can be digested by the amoebae after the fashion of normal amoebae."

Length of life: Numerous fragments with different numbers of nuclei but with the same number of beta granules, crystals, and refractive bodies (Wilber, 1942) were obtained as described above. Great care was taken to have the volume of the various fragments as nearly equal as possible. They were all passed through five

TABLE II

Table showing the relation between the number of nuclei and the length of life in fragments of *Pelomyxa carolinensis* in the absence of food.

Number of nuclei	Number of food-vacuoles	Number of refractive bodies	Average	Length of life in days	Average
1	0	10		8	
1	1	3	6.2	4	6.2
1	0	5		6	
1	1	7		7	
2	2	3		8	
2	0	8	5.6	6	6.3
2	0	6		5	
3	0	4	7.0	5	7.5
3	2	10		10	
4	1	12		6	
4	2	3	6.0	10	8.5
4	2	3		10	
4	0	6		8	
5	1	4		8	
5	9	12	8.0	7	8.3
5	1	8		10	
8	2	12		19	
8	1	8	11.0	23	19.0
8	3	13		15	
10	2	14		17	
10	1	12	12.3	14	18.6
10	3	11		25	
12	1	15	16.0	21	20.0
12	3	17		19	
20	3	20		23	
20	3	19	18.6	19	24.0
20	3	17		30	
25	2	21	18.5	26	23.5
25	1	16		21	
40	2	16	23.0	21	24.0
40	2	30		27	
50	4	18		27	
50	4	10	22.6	29	28.6
50	3	40		30	



FIGURES 2–5.

FIGURE 2. Camera sketch of anucleate fragment of *Pelomyxa carolinensis*. C, contractile vacuoles; F, food-vacuole containing a chilomonad which was ingested after all the nuclei had been removed; F^1, food-vacuole present before the operation; R, refractive bodies; B, beta granules.

FIGURE 3. Camera sketch of a fragment of a pelomyxa one minute after it had been cut off. B, beta granules; C, contractile vacuoles; N, nuclei; R, refractive bodies. The fragment had no contractile vacuoles when it was cut off. Note that one minute later, two contractile vacuoles had formed, but that there was no aggregation of beta granules around them. They contracted about one hour after the sketch was made but there was no indication of aggregation even then.

FIGURE 4. Camera sketch of a fragment of a pelomyxa one hour after it had been cut off. N, nuclei; C, contractile vacuoles; R, refractive bodies; B, beta granules. The fragment had no contractile vacuoles until shortly before the sketch was made. Note that a few beta granules

separate 10 cc. portions of sterile culture fluid, then each was put into 5 cc. of this fluid in a small glass dish and observations made on the relation between the number of nuclei and the length of life.

When the fragments failed to respond to stimulation with a fine glass rod, they were tentatively considered dead. When they showed evidence of disintegration, they were pronounced dead. The occurrence of these two phenomena never differed by more than four hours. The results obtained are given in Table II. This table shows that the fragments with one to 5 nuclei lived roughly the same length of time, but that those with more than 5 nuclei lived much longer.

It may be contended that this is due to differences in the number of refractive bodies and food-vacuoles in the fragments or to differences in their size. As stated above, all the fragments were nearly the same in size. The difference in the length of life observed could therefore not have been due to difference in size. The table shows that while in the fragments with 5 or fewer nuclei the length of life usually varied directly with the number of refractive bodies and food-vacuoles, in those with more than 5 nuclei this did not obtain. This seems to show that while the length of life of fragments of *Pelomyxa* is, at least under some conditions, dependent upon the number of refractive bodies and food-vacuoles present, it is also dependent upon the number of nuclei present.

The evidence presented, then, seems to show that the presence of at least one nucleus is necessary for locomotion and digestion but not for ingestion of food, and that the length of life of a fragment, without food, varies directly with the number of nuclei and the number of food-vacuoles and refractive bodies present.

Formation of the Contractile Vacuoles and the Relation Between Them and the Beta Granules

Numerous pelomyxae were examined under the high power of a binocular dissecting microscope. The animals were left undisturbed on a slide without a cover-slip until they had attached and had extended one or more pseudopods. With the aid of a fine glass rod, pseudopods which contained one or more nuclei, refractive bodies, crystals, and beta granules, but *no contractile vacuoles,* were cut off and mounted on a slide under a cover-slip supported by a ring of vaseline, and examined under the oil immersion objective of a compound microscope.

The following was observed: New contractile vacuoles formed in all the fragments. The time for the formation of new contractile vacuoles varied from about 60 seconds to more than one hour after the operation (Fig. 3). The vacuole was first visible as a small fluid-filled space in the hyaloplasm, no larger than a beta granule. This grew rapidly in size until it was about 5 micra in diameter and then enlarged more slowly until it contracted. At no time was there an aggregation

have aggregated around them. Ten minutes after this sketch was made, the two vacuoles coalesced and another one formed between the two nuclei.

Figure 5. Camera sketch of portion of cytoplasm in a starved pelomyxa twenty-four hours after it had ingested a colpidium. F, food-vacuoles; V, vacuole-refractive bodies in the food-vacuoles; V′, vacuole-refractive bodies in the cytoplasm; B, bleb with a crystal attached to it; C, crystal in a vacuole; R, vacuole-refractive bodies in contact with blebs. Note that some of the food-vacuoles contain crystals and vacuole-refractive bodies. Some of the vacuole-refractive bodies are free in the cytoplasm. Several are in contact with blebs formed from the crystals. Note also two crystals with blebs and crystals in vacuoles.

of beta granules in the region where the vacuole formed but after it had discharged a few times, beta granules aggregated around it (Fig. 4).

The time required for the beta granules to aggregate around the contractile vacuole varied greatly. In some of the fragments studied, there was no sign of aggregation for several days after the vacuole had formed and begun to function; in others, aggregation began very soon after it had formed. The presence or absence of nuclei in the fragments had no apparent effect on the time of formation of the contractile vacuole and the variation in time was as great in anucleate as in nucleate fragments, and the nucleus had no effect on the time required for the aggregation of beta granules around the vacuole.

In uninjured pelomyxae, the cytoplasm immediately around the contractile vacuole is more viscous than that which is elsewhere in the plasmasol (Wilber, 1942). Young contractile vacuoles, however, i.e., vacuoles which had recently arisen *de novo,* were seen often to come into intimate contact with nuclei, food-vacuoles, and other structures in the cytoplasm, and the beta granules in the cytoplasm adjoining them had as marked Brownian movement as those elsewhere in the plasmasol. It was not until after the first contraction that evidence of higher viscosity was observed in the cytoplasm adjoining the surface of the newly formed vacuoles. These facts indicate that the cytoplasm adjoining the surface of the contractile vacuole does not become viscous until some time after the vacuole has arisen. After a viscous layer has formed around a contractile vacuole, the latter always expands in this viscous substance after each contraction. New vacuoles, however, arise elsewhere in the cytoplasm.

It thus appears that in *Pelomyxa* the beta granules are not directly related to the formation of contractile vacuoles and that their aggregation and the gelation of the cytoplasm around the contractile vacuoles are in no way necessary for the action of the vacuoles.

These results are in accord with the contention of Mast (1938), in reference to *Amoeba proteus* but not with those of Howland and Pollack (1927) in reference to *Amoeba verrucosa.* Mast says: "The differentiation of a layer of substance on the surface of the contractile vacuole is probably due to the action of the fluid in the vacuole on the adjoining cytoplasm," and "neither the beta granules nor the layer of viscous substance is involved in the function of the contractile vacuole." Howland and Pollack (1927) maintain that the gelated region around the contractile vacuole can "be considered as supplying the initial impulse for systole. . . ." This is not true in *Pelomyxa carolinensis* for in it the gelated layer does not form until after the first contraction. It is, therefore, not necessarily involved in the contraction of the vacuole and it obviously does not necessarily function in the accumulation of fluid in the vacuole.

It can, therefore, be concluded that neither the beta granules nor the viscous cytoplasm around the contractile vacuoles is of primary importance in the formation or the function of the contractile vacuoles.

Function of the Beta Granules

Wilber (1942) demonstrated that the beta granules in *Pelomyxa carolinensis* stain like mitochondria and he concludes: "These granules, like the beta granules of *Amoeba proteus* (Mast and Doyle, 1935), are consequently similar in composition to the mitochondria in metazoan cells." Metcalf (1910), referring to *Amoeba*

proteus, holds that they are involved in the excretion of substance by the contractile vacuole and he consequently calls them "excretory granules."

Mast and Doyle (1935) attempted to ascertain their function in *Amoeba proteus* by centrifuging and then cutting out different proportions and noting the effect and by observing their movements in normal specimens. They concluded that "the beta granules function in accumulation of fluid eliminated by the contractile vacuole." They are the only investigators who approached the problem experimentally.

In the present work, the experiments and observations of Mast and Doyle were repeated on *Pelomyxa carolinensis*. Some specimens were centrifuged and cut at different levels so as to remove various constituents and different proportions of the beta granules (Fig. 1). Others not centrifuged were cut, to obtain fragments of approximately the same size as the centrifuged fragments, but with normal proportions of all the protoplasmic constituents. Both kinds of fragments were mounted in culture fluid, without food, on slides under cover-slips supported by rings of

TABLE III

Table showing the effect of removing different protoplasmic constituents on the length of life of pelomyxae. F, fat; Cv, contractile vacuoles; H, hyaloplasm; B, beta granules.

Constituents removed	Number of specimens	Average length of life in days	
		Constituents removed	No constituents removed
F + Cv	10	18	21
F + Cv + ½H	9	7	22
F + Cv + H	10	1	19
F + Cv + H + ½B	8	1	20
F + Cv + H + B	8	¼	15

vaseline and studied until all the fragments had died. This was repeated five times. The results obtained in a typical experiment are presented in Table III.

Table III shows the following: The uncentrifuged fragments lived, on an average, from 15 to 22 days. The centrifuged fragments produced by cutting so as to remove the fat and contractile vacuoles lived nearly as long as the uncentrifuged fragments. Those produced by cutting so as to remove the fat, contractile vacuoles, and about half of the hyaloplasmic layer lived about one-third as long. Those produced by cutting so as to remove practically all the hyaloplasmic layer lived only about one-twentieth as long, but those produced by cutting so as to remove approximately half of and all the beta granules respectively showed no marked further decrease in length of life.

It is evident from these results that the removal of the fat and the contractile vacuoles causes but little, if any, decrease in length of life, that removal of a large proportion of the hyaloplasm, in addition to the fat and the contractile vacuoles, causes a pronounced decrease in the length of life, and that the removal of the beta granules in addition to the hyaloplasm, fat, and contractile vacuoles causes little, if any, further decrease in length of life. These facts indicate that length of life in fragments of *Pelomyxa* is primarily dependent on the amount of hyaloplasm in them, and only secondarily, if at all, on the number of beta granules.

The results are in harmony with the contention of Holter (1936) and Holter and Kopac (1937) that enzymes in amoebae and in marine ova are localized in the hyaloplasm and not in any formed bodies, and also with the views of Just (1939) in regard to the great importance of the ground cytoplasm in cellular function.

The facts presented, therefore, seem to indicate that beta granules (mitochondria) do not play an active part in cell functions and that their observed movements are merely a visible sign of submicroscopic changes in the hyaloplasm.

Formation of Refractive Bodies

About 100 pelomyxae were centrifuged and cut so as to remove most of the fat and all the refractive bodies (the fat was removed so that it could not serve as a source of food). Half of them were then put into each of two dishes containing sterile culture fluid and numerous colpidia added to the fluid in one of the dishes. In about 50 minutes all the specimens had recovered from the "shock" of the operations, as indicated by the facts that they moved normally and responded to increase in intensity of light by cessation of movement.

Two of the 50 pelomyxae without food died 20 hours after the operation; four of them lived until the 7th day; none divided. The average length of life was five days. No vacuole-refractive bodies (Wilber, 1942) or refractive bodies formed in any of them. Three of the 50 pelomyxae with food did not feed and died four hours after the operation. Each of the others ingested one or more colpidia. Twenty-four hours after feeding, there were numerous vacuole-refractive bodies in the food-vacuoles and some in the cytoplasm of every one of these specimens, and some of the food-vacuoles in a few of them contained crystals. These results show that food is necessary for the formation of vacuole-refractive bodies and of refractive bodies, and that the crystals are formed in the food-vacuoles. They also show that removal of the refractive bodies has no apparent injurious effects on pelomyxae. This is in accord with the views of Mast and Doyle (1935) who contend "that the removal of all the refractive bodies does not seriously interfere with the vital processes in *Amoeba*," but is not in accord with the views of Singh (1938) who maintains that specimens of *Amoeba proteus* with the refractive bodies removed appear "as if they were encysted until these bodies are formed," and that "as soon as these bodies are formed, the amoebae regain their normal shape and activity."

Ten pelomyxae were kept in sterile culture fluid for 15 days; then colpidia were added and each pelomyxa examined under low and high magnifications at intervals of one hour. The results obtained in observations on all these pelomyxae are in accord with the following detailed description concerning one which had ingested two colpidia: One hour after ingestion, the two colpidia were dead, but there was no evidence of digestion. Two hours after ingestion, both colpidia stained deeply red with neutral red, but the fluid in the food-vacuoles did not stain; the vacuolar membrane was very close to the pellicle of the colpidia. Three hours after ingestion, the colpidia stained deeply red in neutral red, and the fluid in the vacuoles, orange; the vacuoles had enlarged, probably as a result of fluid passing in from the cytoplasm. Four hours after ingestion, both food-vacuoles had divided; fragments of the colpidia in the four resulting vacuoles stained deeply red; two of the vacuoles contained vacuole-refractive bodies which stained intensely red in neutral red (one

vacuole had three; the other, one). Six hours after ingestion, there were six food-vacuoles present; all contained vacuole-refractive bodies; two contained hyaline nonstaining spherical bodies about 4 micra in diameter and of unknown composition. Twelve hours after ingestion, there were numerous vacuole-refractive bodies in each of the six vacuoles and several crystals in four of them; the other two contained no crystals; the vacuole-refractive bodies varied from 1 micron to about 6 micra in diameter; several were free in the cytoplasm; the manner in which they entered the cytoplasm was not ascertained. Twenty-four hours after ingestion, there were eight food-vacuoles present; five contained vacuole-refractive bodies and crystals, three, only vacuole-refractive bodies; there were numerous vacuole-

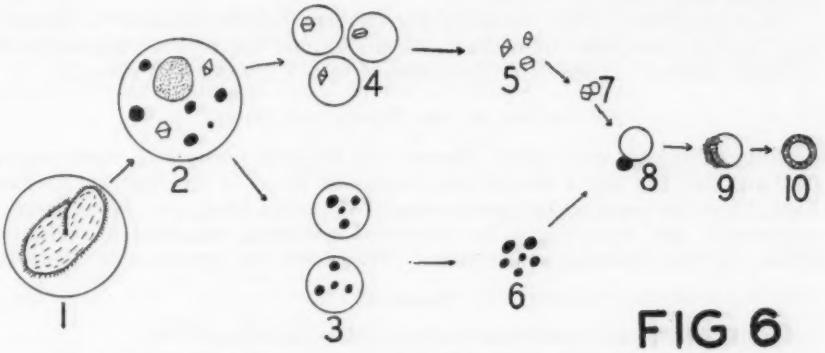


FIGURE 6. Diagram illustrating the formation of the refractive bodies from digested food. 1, food-vacuoles containing an ingested organism, usually a colpidium or a chilomonad; 2, food-vacuole containing a partially digested organism and crystals and vacuole-refractive bodies formed from the food during the process of digestion; 3 and 4, vacuoles formed by the division of 2; 5 and 6, crystals and vacuole-refractive bodies free in the cytoplasm; the manner in which they leave the vacuoles is not known; 7, crystal with bleb attached to it (the crystal disappears and the bleb greatly enlarges); 8, enlarged bleb in contact with a vacuole-refractive body; 9, vacuole-refractive body "flowing over" the bleb; 10, refractive body formed by the union of a bleb and a vacuole-refractive body. Stippling in 9 and 10 represents material of vacuole-refractive body.

refractive bodies and several crystals in the cytoplasm; some of the crystals had blebs attached (Fig. 5).

One of the crystals with a bleb attached was observed closely under oil immersion for several hours. The bleb became larger and simultaneously the crystal smaller until it was no longer visible. Soon another crystal came in contact with the bleb, and in about 40 minutes it too disappeared and the bleb became still larger. The crystals were obviously used in the growth of the bleb. Fifteen minutes later, the bleb came in contact with three vacuole-refractive bodies which were free in the cytoplasm. The substance in these three bodies "flowed over" the bleb and covered it with a layer, resulting in the formation of a body which was identical with other refractive bodies. The time required was 65 minutes. The beta granules took no visible part in the formation of the refractive body. Similar results were obtained in observations on crystals with blebs attached and vacuole-refractive bodies in normal pelomyxae.

The present observations clearly indicate that both the crystals and the vacuole-refractive bodies originate from the food in the food-vacuoles, that they leave the vacuoles and are found free in the cytoplasm (the manner in which this takes place is not known), and that the crystals form nonstaining spherical bodies (blebs) which become covered with a layer of substance from the vacuole-refractive bodies and thus form refractive bodies (Fig. 6).

The conclusions concerning the formation of the refractive bodies from the crystals and the vacuole-refractive bodies are in accord with the contention of Mast and Doyle (1935) who maintain that "The refractive bodies . . . originate and develop in the cytoplasm probably from substances obtained from the vacuole-refractive bodies and the crystals."

Mast and Doyle (1935) maintain that the beta granules function "in transferring digested substances from the food-vacuoles and the crystal vacuoles to the refractive bodies." There is no indication of such a function in *Pelomyxa*.

Function of the Refractive Bodies

Fifty pelomyxae were passed through five different portions of sterile culture fluid and then put into a stender dish containing 25 cc. of this fluid but no food. Each day a few were stained with neutral red, Sudan black, and Janus green B respectively, and these and a few unstained specimens examined for refractive bodies, fat, beta granules, and crystals. The results are presented in Table IV.

Table IV

Showing the decrease in number of refractive bodies in the absence of food.

Date	Number of refractive bodies in each of 9 pelomyxae									Average
	a	b	c	d	e	f	g	h	i	
Sept. 25	50	20	40	50	62	31	23	41	40	39.7
Sept. 30	42	18	32	—	50	27	17	34	30	31.2
Oct. 1	30	10	30	33	50	26	13	30	30	28.0
Oct. 2	23	10	27	27	—	26	13	30	23	22.3
Oct. 3	20	8	27	27	43	—	11	—	23	22.7
Oct. 4	17	8	23	24	43	23	11	25	20	21.5
Oct. 5	14	8	20	20	41	21	11	25	19	19.8
Oct. 10	14	8	17	20	37	15	7	21	10	16.4

This table shows that there is in *Pelomyxa carolinensis* a steady decrease in the number of refractive bodies during starvation. It was observed that some of the refractive bodies disintegrated and that during disintegration the outer layer gradually disappeared, leaving a homogeneous mass which did not stain neutral red. These results indicate that the refractive bodies function as reserve food. This conclusion is in accord with that of Mast and Doyle (1935) and Singh (1938) in regard to the function of the refractive bodies in *Amoeba proteus*.

Summary

1. Centrifugation causes the protoplasmic constituents of *Pelomyxa carolinensis* to stratify into definite layers which, in order of decrease in weight, are as follows:

refractive bodies, food-vacuoles, nuclei, crystals, beta granules, hyaloplasm, contractile vacuoles, and fat.

2. The presence of at least one nucleus is necessary for normal locomotion and digestion, but not for ingestion of food in *Pelomyxa*. The length of life of *Pelomyxa* during starvation varies directly with the number of nuclei present.

3. The contractile vacuoles arise *de novo* in the hyaline cytoplasm. Their formation and functioning is not dependent on the presence of nuclei or beta granules.

4. The beta granules (mitochondria) do not play an active role in cell functions but, probably, merely give visible evidence of submicroscopic changes taking place in the hyaloplasm.

5. The vacuole-refractive bodies and the crystals are formed from the food in the food-vacuoles of *Pelomyxa* and are used in the formation of the refractive bodies.

6. The refractive bodies function as food reserve in the cell. Their complete removal does not impair any of the cellular activities.

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STAGES IN THE DEVELOPMENT OF THE PICKED OR SPINY DOGFISH, SQUALUS ACANTHIAS LINN.

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Introduction

In a previous paper, von Bonde (1945), I described various features of the external development of the oviparous Banded Dogfish, *Haploblepharus edwardsii*. The present species, which attains a length of 915 mm., belongs to the group whose development is classified as ovoviviparous. This species (Plate I, Fig. 1) is very common in the Cape and Natal waters of South Africa and its distribution ranges along the Atlantic coasts of Africa, Europe, and North America and to the Mediterranean.

An egg-case containing three developing embryos (Plate II) was laid by a female in one of the tanks of the Sea Point Aquarium.

Female Reproductive Organs

The only secondary external sexual characters in the female are the pair of cloacal papillae situated one on either side of the median line immediately posterior to the cloacal aperture. The female contains a paired ovary situated in the usual position towards the anterior end of the coelom. These ovaries (Plate I, Fig. 2, r.ov., l.ov.) change in shape and size according to whether the female is gravid or not, although the cycle of the ovarian growth of the ova is such that ripe ova are again in evidence immediately after the young are born. The paired oviducts are not so highly differentiated in structure and function in their anterior half as those of *Haploblepharus edwardsii*, since the ovum or ova is usually enclosed in a very thin transparent chitinous capsule which is not as highly specialized as the egg capsules or mermaid's purses of the oviparous species. The posterior half of each oviduct, as will appear later, is much more highly developed and specialized in conformity with the ovoviviparity of this species. The oviducts meet in the middle line at the anterior end of the coelom and open into the body cavity by a single orifice. By making a transverse incision immediately posterior to the edge of the pectoral girdle and carrying this incision laterally and then anteriorly and lifting the ventral muscular wall, the confluence of the oviducts is exposed. This common opening of the oviducts is attached by a mesentery in the middle line to the surface of the ventral wall of the coelom (Plate I, Fig. 4, f.t.o. and m.). This common opening is wide and funnel-shaped and lies right over the antero-ventral part of the ovaries so that immediately after a ripe ovum escapes from the ovary it is taken up by the lips of the funnel and passed backward along the fallopian tube (Plate I, Fig. 4, f.t.).

Each oviduct may be divided into five distinct portions as follows:

(1) The fallopian tube (Plate I, Figs. 2 and 4, f.t.) is extremely narrow, thin-walled and comparatively short. This portion is followed by (2) the albumen gland (a.g.) which in this species is very small, thick-walled and muscular, the internal surface being richly supplied with glands secreting the albumen which covers the ovum. The albumen gland is followed by (3) the nidamental gland (n.g.) which is a long thin tube of about the same diameter as the fallopian tube. Here the transparent chitinous egg capsule is secreted. The nidamental gland opens posteriorly into (4) a very wide saccular uterus (u.) in which the development of the embryo takes place. The uterus in turn narrows into (5) a short vagina (v.) which opens into the cloaca. In immature females the vaginal orifices are separated from the cloaca by a membrane or hymen which during copulation is pierced by means of the special claws of the claspers (see below). It is interesting to note that in those Elasmobranchs in which the shape of the claspers of the male is flat, the vaginal orifices are slit-shaped, while in those forms in which the claspers are provided with hooks, the lining of the vagina is thickened (Daniel, 1934).

The uterus, which was described in detail by Widakowich (1907a), is more or less typical of those species displaying viviparity or ovoviviparity. In *S. acanthias* each uterus is a greatly enlarged sac with well defined boundaries. The mucous membrane of each uterus varies greatly in conformity with the development of the ovaries. Thus, when the ovaries are large and contain ripe ova ready for fertilization, the mucous membrane of the uterus is more or less smooth. In gravid females, on the other hand, this mucous membrane is thrown into longitudinal rows of flaps known as papillae or villi, each of which is a leaf-like structure with very thin walls (Plate III, Fig. 10, p.). The border of each papilla is thickened by a blood vessel. The blood supply to the uterus is exceedingly profuse (Daniel, op. cit.). The arterial supply consists in part of the anterior and in part of the posterior oviducal arteries which break up into branches to the rows of papillae. Each terminal artery runs along the free borders of the papillae to supply each single papilla with blood. The blood from each papilla is drained by central veins into a main uterine vein. The two systems are connected by a capillary net around the surface of the papilla.

Ercolani (1879) described the viviparous Selachians according to the various aspects of the relationships between the developing eggs and the inner wall of the uterus, and, according to the degree of these relationships, he differentiated four classes:

1. Naked contact between the homogeneously smooth mucous membrane of the uterus and the surface of the egg (*Plagiostomi acotyledonale*). *Squatina angelus* and *Heptanchus cinereus* are cited by him as examples.

2. Complicated contact between both surfaces, the complication being due to the presence of numerous folds in the secreting mucous membrane of the mother which causes an appreciable increase of the inner surface (*Plagiostomi acotyledonale*). *Squalus acanthias* and *Scymnus lichia* are cited as examples.

3. The complicated contact is differentiated by a more or less significant new structure of papillae on the folds of the secreting mucous membrane of the uterus by means of which its inner surface and activity are extraordinarily enlarged (*Alcuni selachii*). *Torpedo* and *Pteroplatea micrura* are cited as examples. (Note: According to the development of the papillae in the uterus it appears as though *S. acanthias* should be placed in this group rather than in Group 2.)

4. The relationship is no longer due to contact but to an intimate union between a part of the absorbing upper surface of the egg with a part of the secreting surface of the uterus. Both surfaces form folds which interlace and at the same time fuse with each other. Thus a type of rudimentary placenta is formed which is comparable with the mammalian placenta (*Plagiostomi cotyledonale*). *Carcharias glaucus* and *Mustelus laevis* are cited as examples.

In *S. acanthias* the papillae of the mucous membrane arise from a cutaneous development of the convoluted marginal arteries. The papillae are twisted around their longitudinal axes. The changing form of the papillae is due partly to a purely mechanical and partly to a reflex action. There appears to be no relationship between the gestation period and the length of the papillae. The uterus is supplied with blood from two capillary systems, viz., (a) the organotropic and (b) the embryonotropic. The latter forms a capillary network which covers the papillae and also the remaining part of the inner wall of the uterus. The capillary network receives its blood from the marginal arteries and releases it into the basal veins. These do not possess any separate musculature but are embedded in the muscular fasciculi of the uterus which open towards the papillae.

Widakowich (1907b) described the closing device ("Verschlussvorrichtung") situated between the portion I have designated as the nidamental gland and the dome of the uterus (Plate I, Fig. 2A, 2B, c.d.). The gravid uterus (Plate I, Fig. 2, u.) has a cylindrical shape with conical ends pointing anteriorly and posteriorly. The uterine arteries are strong walled (Plate I, Fig. 2A, a.) and run laterally along the nidamental glands. The uterine vein (ve.) runs in close proximity to the artery. The closing device is necessary since the uteri, as soon as the embryos reach a definite age and their enveloping membranes rupture, are filled with a large quantity of nutrient yolk, which, but for this closing device, would be forced along the whole length of the oviducts during any contraction of the uterus, or external pressure from the distension of the alimentary channel, or the movements of the embryos in the uteri should the yolk sac be ruptured. During the time of rut and fertilization there is a strong hyperaemia of the whole genital tract; hence the genital tract is well supplied with blood vessels. The function of the papillae above referred to will be dealt with later.

Male Reproductive Organs

The morphology of the male reproductive system follows the general plan of all Elasmobranchs. The testes are paired and occupy the anterior half of the

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Plate I

Figure 1. Left lateral view of *Squalus acanthias*.

Figure 2. Dissection of a gravid female showing the reproductive organs.

Figure 2A. Closing device (c.d.) in oviduct (after Widakowich).

Figure 2B. Internal surface of the dome at the anterior end of the uterus showing the closing device. The dome has been invaginated to bring the closing device into prominence, hence it appears nipple-shaped.

Figure 3. Egg capsule containing single ovum in early stages of development.

Figure 4. Portion of the anterior ends of fallopian tubes (f.t.) showing their confluence and coelomic orifice (f.t.o.).

Figure 5. Clasper of male.

Figure 6. Claw (cl.) and spur (sp.) of clasper greatly enlarged.

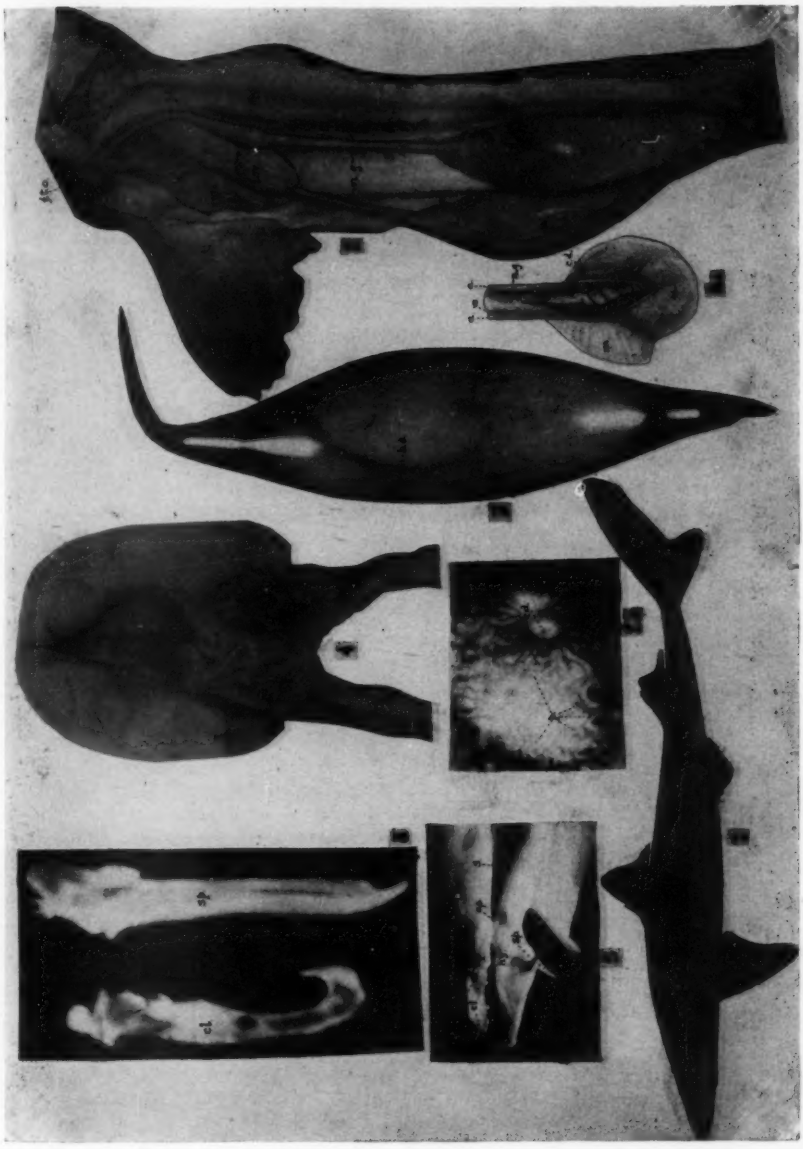

PLATE I

coelom. From the anterior end of each testis the vasa efferentia lead to the epididymus which is much coiled. The epididymus eventually widens out into the vas deferens which expands and joins its fellow in the middle line posteriorly, opening into the cloaca by a single pore.

The external secondary sexual characters of this species present certain distinctive features. As in all Elasmobranchs the basal element (basipterygium) of each pelvic fin is prolonged as a stout, backwardly-directed rod, sharply demarcated from the remainder of the fin, forming an intromittent organ, called the clasper or myxopterygium. A transverse section of the proximal half of the clasper shows it to be almost completely round, the internal cartilaginous skeleton forming a tube filled with a thick muscular substance through the center of which passes a duct. In the distal end the skeleton only occupies the ventral part of the clasper, being continued right to the tip. The latero-dorsal wings of this part of the clasper are formed of thickened skin, the outer wing being folded over the curved inner wing to form a canal which is continuous with the above-mentioned duct in the proximal half. This duct opens through the hypopyle into an open channel which is continued to the tip of the clasper (see Plate I, Fig. 5, hy.). On the outer surface near the hypopyle, a strong spiny spur (sp.) arises. On the inner ventral surface a claw (cl.), which extends to the tip of the clasper, is situated. This claw is much curved and, like the spur, is movable. Its function is probably to rupture the hymen. By pressing the distal end of the clasper, the spur is erected, and forms an important organ of attachment, penetrating and even lacerating the tissues of the female (Leigh-Sharpe, 1920). The spur in its retracted position lies alongside the claw in the same groove. The rhipidion (r.) in the normal intromittent position of the clasper covers the dorsal part of the groove and also the claw and the spur. The siphon (s.) is comparatively small and is situated immediately beneath the skin of the pelvic fin. The apopyle (ap.) lies some distance from the cloaca and relatively near to the hypopyle.

Development

It was not possible to study the complete development of the present species along the lines described in my previous paper (op. cit.) and it is, therefore, necessary to refer to the description of embryos of various lengths taken from gravid females. The gravid uteri shown in Plate I, Fig. 2, u. each contained a single egg capsule with a single ovum (Fig 3.), one being infertile.

A brief description of the primary phases in Selachian development is as follows: At the time of fertilization the ovum is usually in the shape of a large spherical mass, similar to the yolk of an avian ovum, but its shape may become roughly cylindrical as in the present case, the shape being determined by that of the enclosing egg capsule. The greater part of the egg is formed of peculiar oval spherules of food-yolk held together by a protoplasmic network (Balfour, 1881). The yolk is completely covered by the albumen which is thick and glairy and transparent except at the polar extremities. The protoplasm is concentrated in a small lens-shaped area, the germinal disc or "orange spot," and is situated much nearer to the broader end of the egg. Yolk spherules are also present in this disc, but are much smaller and of a different character. Segmentation is meroblastic or discoidal as in all telolecithal eggs, being confined to the germinal disc.

The blastoderm is thus formed and at the close of segmentation the blastoderm forms a lens-shaped disc thicker at one end than the other, the thicker end being known as the embryonic end where the future embryo develops. The three germinal layers become established and shortly after this the rudimentary embryo consists of an oblong plate which extends inwards from the periphery of the blastoderm. This plate is the medullary plate and along its axial line a shallow groove, the neural groove, is formed. The embryo now progressively increases in length and a certain number of mesoblastic somites become formed. The increase in length of the body takes place normally by growth in the region between the last mesoblastic somites and the end of the tail. The anterior part of the body is not completely folded off from the blastoderm and the neural groove becomes converted into the neural canal. As soon as the embryo has become folded off from the yolk both in front and behind, the separate parts of it are easily recognizable. The embryo is attached to the yolk by a distinct stalk, which gradually narrows and elongates and is known as the umbilical cord. The blastoderm continues to grow in all directions and by circumcrescence it provides a protective vascular covering of the yolk mass, but these extra embryonic structures will eventually be resorbed. The protective covering is developed from the ectoderm, whilst the vascular covering arises from the mesoderm. Before completely covering the yolk mass, however, at a certain stage a yolk blastopore, which is that portion of the surface of the yolk mass not yet covered by the blastoderm, is formed. The yolk blastopore is related to gastrulation since circumcrescence is an important factor in gastrulation and it assists in laying down the definitive endoderm. On the ventral surface of the head of the embryo the stomodaeum is formed, which eventually gives rise to the mouth, while some little distance from the posterior end of the embryo the proctodaeum will eventually give rise to the anus. At a somewhat later stage the gill clefts are formed, being at first six pairs in number, but soon the anterior pair becomes separated from the rest to give rise to spiracles. The first rudiment of the heart becomes visible during the 38 somite stage and has a cavity situated between the mesoderm and the endoderm. All the visceral clefts, including the spiracles, show long filiform external gills which project out for some distance from the sides of the head. These external gills lie against the front wall of the spiracle and the front walls of the visceral clefts. It appears as though they are specially developed to facilitate respiration during the earlier stages of development, either intra-uterine or intracapsular. They disappear some time before the close of the embryonic stage. The young Selachian has all the external characters of the adult when hatched, and can immediately swim about and fend for itself, but before hatching, the yolk has been completely absorbed by the developing embryo and the yolk sac and umbilical cord decrease in size until eventually they are only shown in the form of an umbilicus which persists for about 14 days after birth. The vitelline circulation becomes developed at a stage before the closure of the yolk blastopore. During the circumcrescence of the blastoderm its edge has remained thickened and this feature persists until the whole of the yolk is covered. In this thickened edge a circular vein arises which brings the blood back from the yolk sac to the embryo. The first stage in the development of the vitelline circulation is the formation of a single artery which emerges from the umbilical cord and proceeds cephalad along the blastoderm under cover of the head of the embryo. This arterial trunk divides to form two arcuate branches that turn laterad and then posterad towards the posterior margin

of the blastoderm. These arterial branches eventually meet posteriorly and give off numerous small secondary branches, some of which connect with small veins emptying into a venous ring close to the margin of the blastoderm. The main trunk of the vitelline vein drains the venous ring and runs straight to the umbilical cord (Smith, 1942). When the yolk blastopore has completely closed, the venous ring disappears and the area formerly occupied by the yolk blastopore is traversed by a continuation of the main trunk of the vitelline vein. This trunk receives numerous small veins usually joining it at right angles. Since the venous trunks are only developed behind the embryo, it is only the posterior part of the arterial ring which gives off branches. At a still later stage the arterial ring embraces the whole yolk, but later vanishes in its turn, as did the venous ring before it. There is then present a single arterial trunk and a single venous trunk. The arterial trunk is a branch of the dorsal aorta, and the venous trunk enters the heart together with the splanchnic vein. The venous trunk enters the body on the right side and the arterial leaves on the left of the umbilical cord.

The yolk sac persists during the whole of the embryonic life and in the majority of Elasmobranch embryos there arises within the body walls an outgrowth from the umbilical canal into which a large amount of the yolk passes. This outgrowth forms an internal yolk sac. In viviparous species a remarkable peculiarity arises in that the vascular surface of the yolk sac becomes raised into a number of folds which fit into corresponding depressions in the vascular walls of the uterus. The yolk sac becomes in this way firmly attached to the walls of the uterus and the two together constitute a kind of placenta (Balfour, op. cit.).

We may now proceed to a description of the various embryos obtained from gravid females:

1. The earliest stage found is that shown in Plate I, Figure 3. The large ellipsoidal yolk mass, having an axial length of 58 mm., is shown enclosed in the transparent egg capsule which had an overall length of 100 mm. The oval shaped blastoderm, measuring 29 mm. along its longer axis, lies on the posterior half of the yolk and at the rounded anterior end it shows the formation of the neural groove (ne.g.). The thickened edge of the blastoderm (b.e.) is well shown. The egg capsule is composed of an extremely thin membrane which is wholly transparent except at the polar regions where the capsule tapers to a sharply pointed end posteriorly and a blunter pointed end anteriorly. These ends are designated anterior and posterior in relation to the position of the embryo and not in regard to the position the capsule takes in the gravid uterus. The figure shows the capsule in the position in which it lies in the uterus, the blunter anterior end being directed posteriorly in relation to the female. The yolk has a thin covering of glairy albumen over the whole surface, but towards the poles the albumen is much thicker and becomes more or less opaque and is loosely attached to the capsule. Encapsulated eggs, which were removed from the uteri, were placed in a large pneumatic trough with running sea water. After a few days the capsules invariably ruptured around the equator and albumen was only found in the pointed polar ends. Compared with the egg cases of oviparous species, the egg capsules of *S. acanthias* contained very little albumen.

2. Plate II shows an egg capsule containing developing triplets (em. 1, 2, 3). These embryos were about 40 mm. in length, the total length of the capsule being

180 mm. The yolk was rounded in conformity with the shape of the enveloping capsule. All attempts to keep these embryos alive after the extrusion of the capsule failed. Embryo 2 ruptured its yolk sac and died, the remaining two embryos also succumbing in rapid succession.

3. The next stage is shown in Plate III, Figures 7A and B. These embryos were taken from a gravid female which had a single egg capsule in each uterus, each capsule containing a set of twins. Immediately after the female was opened, the capsules were removed from the uteri and placed in running sea water in a pneumatic trough. It was not possible to determine their age at this stage. After three days, one capsule ruptured and the embryos freed themselves from their enveloping membrane and lived for about twelve days. The twins in the other capsule died after five days and it is interesting to note that then they were smaller than their counterparts, although at the time of removal from the uteri, all the embryos were of equal size, viz., 45 mm. in length. The longitudinal diameter of the yolk sac at this time was 80 mm. The live embryos, a week later, had attained a length of 65 mm. The longitudinal diameter of the yolk sac was now 70 mm., thus showing

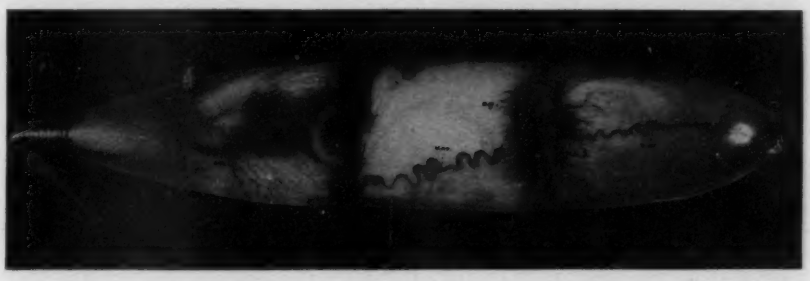

PLATE II

Egg capsule containing three developing embryos.

a corresponding decrease as the embryo grows. The umbilical cord (u.c.) was relatively long and hollow, forming a tubular connection between the yolk sac and the intestine of the embryo. By a gentle pressure on the yolk sac it was possible to force particles of yolk along the umbilical cord into the embryo's intestine. Its walls were very thin and laterally in them ran the vitelline vein (v.v.) and artery. The internal anatomy was well developed at this stage, the alimentary canal being completely formed with a spiral valve developed in the valvular intestine. The liver was developed and the heart had assumed the typical Selachian form. The external gills (Figs. 8 and 9, e.g., sp.g.) had reached their maximum development. After twelve days the other pair died and the external gills were then beginning to shrivel up.

4. A more advanced embryo is shown in Plate IV, Figure 12. Here the external gill filaments had disappeared. The yolk sac was now relatively small and the umbilical cord short and thick. The embryo had assumed the typical adult form except for the yolk sac.

5. The most advanced stages of development found in a gravid female occurred

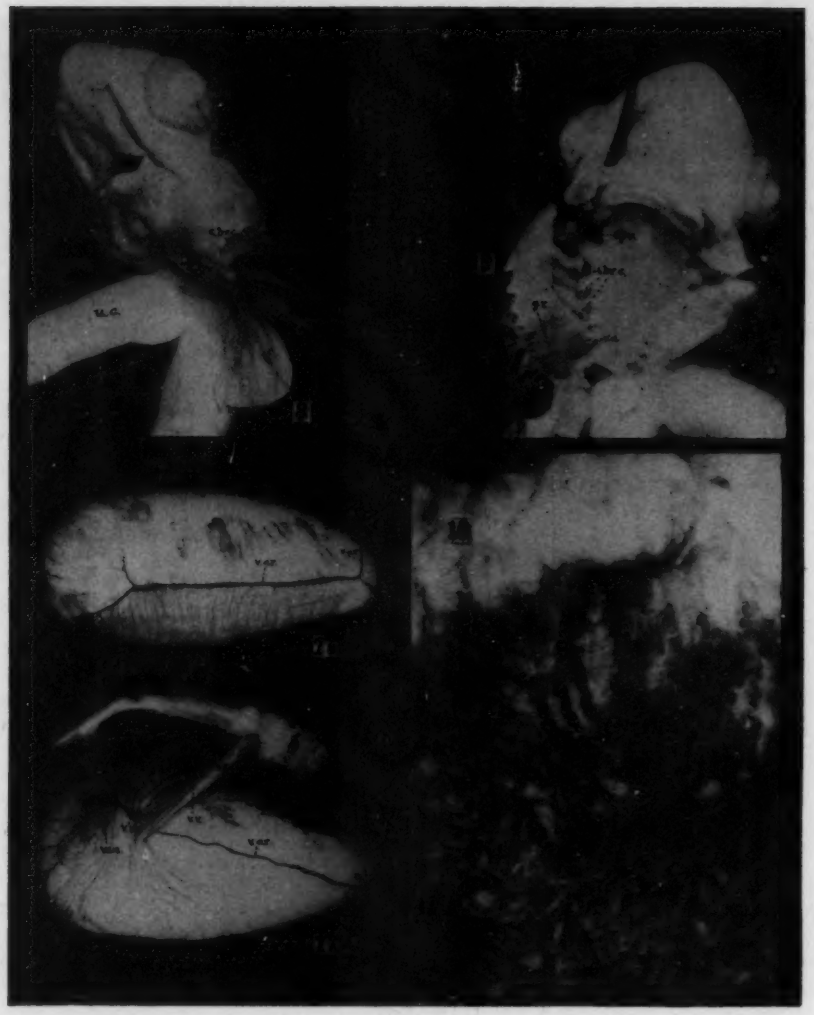

PLATE III

FIGURE 7A. A 70 mm. embryo showing the vitelline circulation and the attachment to yolk by the umbilical cord (u.c.).

FIGURE 7B. The hemisphere opposite to the one to which this embryo is attached.

FIGURE 8. Cephalic end of the same embryo showing the external gill filaments (e.g.) and the spiracular gill filaments (sp.g.).

FIGURE 9. Dissection of the buccal cavity of the same embryo showing the external gill filaments attached to the visceral arches (v.a.). The internal branchial clefts (i.br.c.) are also shown.

FIGURE 10. A portion of the internal surface of the uterus, much enlarged, showing the papillae (p.).

in a female 800 mm. in length. Here the six embryos were at the point of birth. They were 220 mm. in length and differed from the one shown in Plate IV, Figure 12 principally in the fact that the yolk had been completely resorbed and the remaining yolk sac only 5 mm. in length. The embryos had the same coloration as the adults. It was not possible to determine the age of these embryos so that up to the present the gestation period is unknown.

PLATE IV

FIGURE 11. Entire embryo of 100 mm. attached to the yolk sac.

FIGURE 12. More advanced embryo 140 mm. in length. The dotted line at the proximal end of the umbilical cord (u.c.) shows the relative size of the yolk sac in a 220 mm. embryo.

ABBREVIATIONS

a. uterine artery, a.g. albumen gland, ap. apopyle, b.e. edge of blastoderm, c.d. closing device, cl. claw, clp. clasper, e.br.c. external branchial cleft, e.g. external gill filaments, em. 1, 2, 3. developing embryos, f.t. fallopian tube, f.t.o. coelomic orifice of same, g.r. gill rakers, hy. hypopyle, i.br.c. internal branchial cleft, l.ov. left ovary, m. mesentery, n.g. nidamental gland, ne.g. neural groove, ov. ovary, p. papillae, r. rhipidion, r.ov. right ovary, s. siphon, sp. spur, sp.g. spiracular gill filaments, spi. spiracle, u. uterus, u.c. umbilical cord, v. vagina, v.a. visceral arch, v.ar. vitelline artery, v.v. vitelline veins, ve. uterine vein, ven. venules, y.s. yolk sac.

External Gills

Gudger (1940, pp. 626, 627) writing about external gill filaments in shark embryos states—"The embryos of all nonplacental viviparous sharks and rays known to me have long external gills. The eggs of these Elasmobranchs have thin diaphanous shells, through which uterine fluids readily penetrate. These fluids are milk-like secretions of the uterine mucosa and serve as food for the growing embryos, which absorb this food through their long filamentous gills. It has been indicated that the relatively thick shells of *Chlamydoselachus* are burst by the growing embryo, are cast off into the uterus, and are then or later thrown out into the sea. Two investigators (Hawkes, 1907 and Smith, 1937) have found highly vascularized areas in the wall of the right uterus (cf. my Plate III, Fig. 10). These observations suggest that these areas might have served to secrete foodstuffs into the uterus. Then the long gestation period and the enormous size of the relatively late embryos still attached to large yolk sacs seem to indicate that these embryos grow not at the expense of the yolk alone. All these things lead to the inevitable question—'Do the external gills of the embryos of *Chlamydoselachus* serve to absorb food from a uterine secretion?' . . . It seems quite sure that in any case these external gills of the non-extruded juvenile sharks serve as respiratory organs."

Further, Gudger (op. cit., pp. 559–562) in dealing with the matter of intrauterine gestation and respiration states (p. 561) "on the whole it seems quite probable that the young of both sharks (*Ginglymostoma* and *Chlamydoselachus*) may receive some oxygen by diffusion from the uterine wall into the fluids surrounding the embryo. Furthermore, from my knowledge of uterine gestation in other sharks and rays, I am strongly of the opinion that the uterine wall in both (sharks) secretes liquid food materials to nourish the young after they are freed from the egg capsules. . . . The long external gills of embryos of rays and of other sharks, when bathed in the uterine fluid, may take in not only oxygen but mineral salts and possibly other food substances as well. The rich plexus of vitelline capillaries will also be bathed in the fluid of the uterine cavity and they may absorb some food and oxygen from it. If this takes place in *Chlamydoselachus,* it must go on for a long time, until and even after the yolk is resorbed, and this yolk must be used up before the fish is born, else the free oceanic life of this little shark would be very brief."

There are many points of similarity between the present species and that described by Gudger. *Squalus acanthias* is ovoviviparous and at a certain stage of incubation the egg capsule bursts and the embryo is free to lie in the uterus. I have dissected gravid females in which parts of the capsule were found in the uterus. Plate IV, Figure 11, shows an embryo which lay completely free in the uterus with the remains of the capsule lying near the vagina, evidently ready to be extruded. The fact that it has been possible for me to keep some embryos of this species alive in sea water for some time after their removal from the uterus seems to indicate that the external gills must have a function purely respiratory. It is of interest to note that in all dissections of the uteri of gravid females the embryos lay with their heads pointed towards the cloaca and this leads me to infer that the relatively wide vaginal openings into the cloaca permit of sea water entering the uteri for the respiratory functions of the growing embryo. (Compare the provision of respiratory slits in the mermaid's purses of oviparous sharks through which water can enter to provide

respiration by means of the external gills—von Bonde, op. cit.) The external gill filaments are doubtless precocious growths of normal gills and they are eventually lost during later development. Compare Figures 11 and 12 which portray embryos of greatly varying stages of development. In the latter figure the external gill filaments have completely disappeared and the functional gills are typically adult in structure. The occurrence of external gill filaments protruding from the spiracular cleft is conclusive evidence that the spiracular cleft in sharks was primarily a gill cleft with a respiratory function.

Widakowich (1907a) states that the external gills are lost as soon as the egg capular membrane disappears. This is contrary to my observations on embryos kept alive artificially in sea water, and also on those which had burst the egg capsule and were lying free in the uterus, but he does corroborate the view that these transitory gills do not serve the purpose of taking up nutrient material.

There is an alternative possibility in connection with the respiration of the embryo during the time that it has the external gill filaments. In no case has it been possible to observe any connection between the mucous lining of the uterus and the yolk sac even faintly resembling a placenta. This occurrence of a placental development in *S. acanthias* is much featured by Widakowich (op. cit.), but it seems more probable that the papillae of the uterus, which are richly supplied with blood vessels, can, by a process of osmosis, aerate the blood of the developing embryo through the capillary system in the external gill filaments. As mentioned before, Ercolani (op. cit.) grouped this species under his second group of uterine developments and he mentioned the fact that these papillae are in close contact with the vitelline membrane of the yolk. I am convinced, however, that at no stage during the intra-uterine development of the embryo does the mother play any part in the nourishment of the embryo by means of the external gill filaments, the yolk sac alone functioning in this capacity. This contention is, moreover, strengthened by the fact that the yolk sacs in all gravid females dissected by me never showed any convolutions into which the vascular papilla of the uterus could fit. There is of course a possibility that here also there may be an osmotic transference of blood from the surface vascular papillae to the vitelline circulation, but I cannot see that such a function is necessary to the developing embryo. I, therefore, lean to the view that the sole function of these vascular papillae is to provide aeration through the external gill filaments, this function being in addition to that previously described where such aeration takes place by means of the sea water entering the uterus.

The Vitelline Circulation

The earliest stages of the yolk sac circulation are shown in Plates II and III, Figures 7A and 7B. The vitelline blood vessels finally spread out over the whole yolk sac. Their function is to carry the nutrient yolk to the developing embryo. The early stages of the circulation were briefly described above. In all young embryos of *S. acanthias* a vitelline artery arises from the dorsal aorta and runs along the left side of the umbilical cord and thence forward from the yolk stalk (u.c.) over the surface of the yolk sac (v.ar.). In earlier embryos, this artery is sinuous (Plate II, v.ar.) but later it becomes straightened out. Its usual course runs from the umbilical cord to the anterior sharper apex of the yolk sac but there

are variations where it runs obliquely across the yolk sac to the sides. In every case, however, it curves over the yolk sac and branches into two, one arm being short whilst the other passes straight back along the ventral surface of the yolk sac (Fig. 7B, v.ar.) and then forks into two branches. On this ventral surface the vitelline artery gives off numerous side branches at right angles to itself whilst no branches are seen arising on the dorsal surface of the yolk. Posteriorly, on the dorsal surface there is a single main vitelline vein (Fig. 7A, v.v.) which drains a dendritic group of venules (ven.). This main vitelline vein runs along the right side of the umbilical cord and enters the heart with the splanchnic vein. In its course along the umbilical cord the main vitelline vein is joined by smaller veins near the yolk sac.

Summary

1. *Squalus acanthias* is an ovoviviparous dogfish with a wide range of distribution.

2. The male and female reproductive systems, especially the internal structure and the closing device of the uterus, are described.

3. Oviparous development takes place in a thin transparent egg capsule, different from the usual Selachian egg capsules, up to a certain stage, when the capsule ruptures and then the development becomes viviparous.

4. Embryos at different stages of development are described. Some capsules contained single embryos, others twins and still another triplets.

5. The functions of the external gills are dealt with, it being concluded that their function is primarily respiratory.

6. The vitelline circulation is described.

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EMBRYONIC DEVELOPMENT IN POECILIID FISHES

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In *Heterandria formosa,* a viviparous cyprinodont fish of the family Poeciliidae, nearly all of the nourishment for embryonic development is obtained from the mother after fertilization through a pseudoplacental association (Scrimshaw, 1944). Similar although less complicated associations between mother and embryo exist in other species of this family (Turner, 1937, 1940). These species are for the most part considered to be ovoviviparous. The distinction is based on the difference between live bearing forms which retain an egg with a full supply of nourishment for development and those whose embryos receive nourishment from the mother. The latter are considered truly viviparous.

Turner (1937) referred to all species of Poeciliidae as ovoviviparous. He obviously used this term in a general sense for he suggested in discussing the data of Bailey (1933) that *Xiphophorus helleri* could receive nutriment from the parent. Turner also pointed out (1937) that the small *Heterandria* egg cannot contain enough nourishment to account for the size of the larvae and suggested that the follicle cells surrounding the embryo furnish food materials.

The weight values reported by Bailey for various embryonic stages in *Xiphophorus* show no decrease in the weight of the total yolk-embryo system. Since energy is used for maintenance metabolism, the total weight of this system decreases in forms depending entirely on yolk. Gray (1928) reported a decrease of 37 per cent for the oviparous trout, *Salmo fario,* and Hsiao (1941) found a decrease of 34 per cent in the truly ovoviviparous perch, *Sebastes marinus.* Accordingly, although he does not suggest this, Bailey's data show that some nourishment must be obtained from the mother by the developing embryo of *Xiphophorus.*

It is true that most poeciliid fishes are more dependent on the yolk laid down before fertilization than upon maternally supplied nourishment after that time. However, the evidence presented below shows that the members of this family do utilize nourishment outside of that contained in the yolk and hence are not ovoviviparous in the strict sense of the term. Embryos of such species as *Heterandria formosa* and *Aulophallus elongatus* are truly viviparous and as dependent on the mother for nourishment as are those of a placental mammal.

Gray (1926, 1928) reported the relationships between nourishment and growth rate in the oviparous trout, *Salmo fario.* The problems of oviparity and ovoviviparity are similar in that in each the embryo has its own supply of nourishment and receives food and water from its environment. When these conditions are compared with true viviparity, striking differences are noted (Scrimshaw, 1944). The present study was undertaken to find and describe intermediate stages between

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viviparity and ovoviviparity in other members of the family. Such stages would aid in the understanding of the complex problems of viviparity in this specialized group. The weight changes in the egg and embryos of twenty additional species of Poeciliidae have been determined and a condition intermediate between ovoviviparity and true viviparity characterized. There appeared a remarkable tendency for the young of all the species examined to receive from the mother only as much in terms of dry weight as they required for the maintenance of tissues already formed.

Material

Stocks of the following species were maintained in the laboratory and the gravid females were available as desired. These were dissected and the living embryos weighed.

Gambusia affinis holbrookii
Lebistes reticulatus
Poecilistes pleurospilus
Quintana atrizona
Xiphophorus helleri

The following species were collected in the field and the embryos weighed at the Institute for Research in Tropical America, Barro Colorado Island, Canal Zone (June–August, 1940).[2]

Allogambusia tridentiger
Aulophallus elongatus
Brachyrhaphis cascajalensis
Brachyrhaphis episcopi
Darienichthes dariensis
Mollienisia sphenops

In addition the writer is greatly indebted to Dr. Carl Hubbs of the Museum of Zoology, University of Michigan, for preserved material of the following species:

Allophallus kidderi
Belonesox belizanus
Brachyrhaphis terrabensis
Dextripenis evides
Gambusia nobilis nobilis
Mollienisia velifera
Poecilia vivipara
Poeciliopsis infans
Poeciliopsis occidentalis
Poecilistes pleurospilus
Pseudoxiphophorus bimaculatus taeniatus

Through the kindness of Dr. William Schroeder, additional specimens of *Gambusia affinis holbrookii* were obtained from the Museum of Comparative Zoology of Harvard University.

The gravid females of all of the above species were dissected and the contents of the ovary noted. In all of these forms the embryos are retained within the follicles of a large single median ovary which was removed intact before examination.[3] The embryos could be dissected out with the ovisac intact and the ap-

[2] This work was made possible by a grant from the Penrose Fund of the American Philosophical Society and the assistance of Dr. Thomas Barbour of the Museum of Comparative Zoology, Harvard University. The author was greatly assisted in the field work and weight measurements by Mr. Thomas C. Saunders of Harvard University. The cooperation of Mr. James Zetek, custodian of the island and laboratory; Mr. Graham Fairchild of the Gorgas Memorial Laboratory, Panama; and Mr. John Wood of the Canal Zone Police considerably aided this work.

[3] Dissection and weight data for all of these species are contained in the appendix of the author's doctorate thesis *Embryonic Growth in Poeciliid Fishes* deposited in the Harvard University Library.

proximate diameter measured with an ocular micrometer. The weight determinations were made as described for Heterandria (Scrimshaw, 1944), the embryos being dried on small cover slips over calcium chloride and weighed on a Sartorius balance. Any error in the actual dry weight recorded was insignificant in comparison with the variability of the material. Wet weights are not discussed because of their variability in preserved material and the difficulties in determining excess fluid. The taxonomic scheme followed throughout is that proposed by Hubbs (1924, 1926).

Experimental

Dry weight determinations of the eggs and embryos in the intact ovisac were made at various stages between fertilization and parturition. In each case as many stages as could be obtained were studied and ranked according to age as described below for purposes of graphical and statistical representation.

It was soon observed that these weights were within the same general range regardless of the stage of the embryo. When least square lines were calculated through these points, a straight line parallel to the abscissa served to describe the data. Figures 1 and 2 show representative development lines and the scatter of weight values for four of these species.

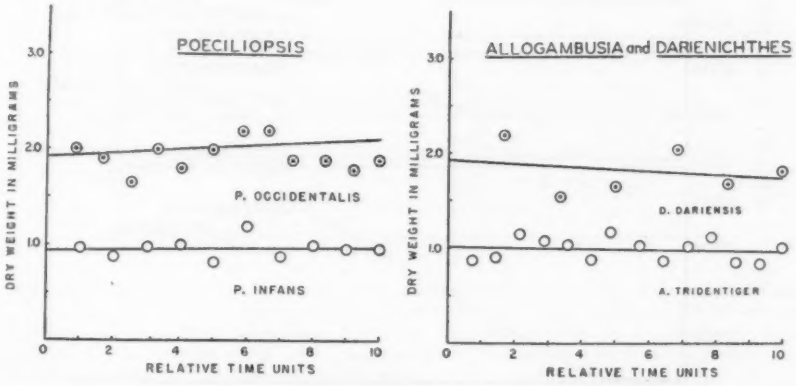

Figure 1. The dry weights of the embryos in the intact ovisac are arranged on a relative time axis in order of stage of development. For graphical and statistical purposes it has been necessary to assume that the time between stages is equal in all cases. Fertilization is represented at the beginning of the time scale and parturition at the end. The lines are calculated through these points by the method of least squares.

Figure 2. Two closely related species are portrayed in the same manner as those in Figure 1.

On the basis of these straight lines, the mean weight of the embryos of this group of poeciliid species can be compared (Figs. 3 and 4). The range is so great that all of the species could not be represented in one figure, but several forms are repeated in Figure 4 to facilitate comparisons. In addition to the species described

in this paper, *Heterandria formosa* and *Aulophallus elongatus*[4] are included as examples of viviparous poeciliid fish securing nearly all of their nourishment from the mother. The straight line for *Aulophallus* is purely diagrammatic, but its use

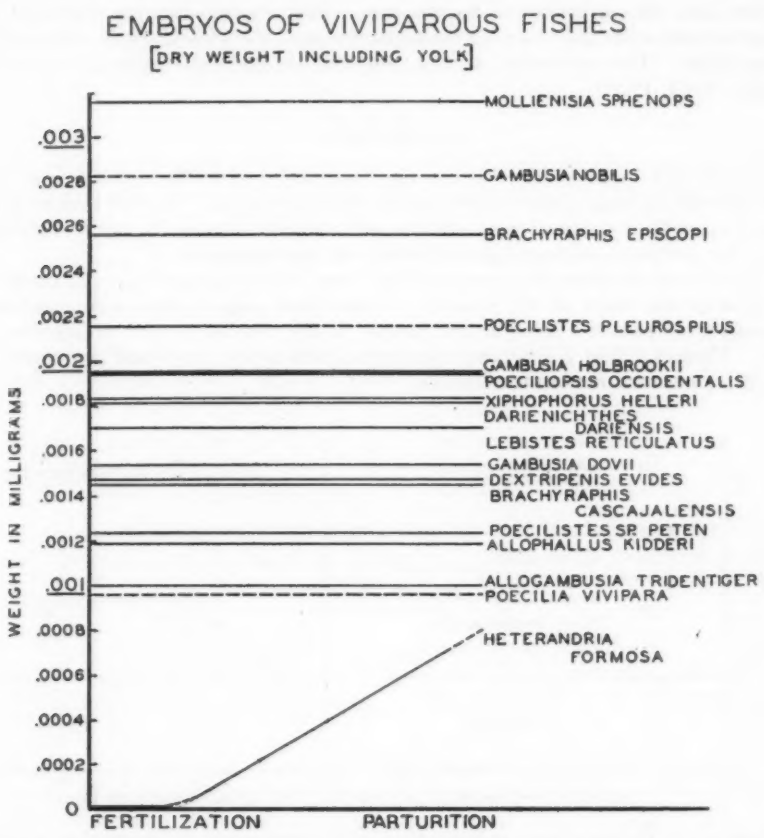

FIGURE 3. For purposes of comparison the dry weight curve of the embryo during development is drawn as a horizontal line. The average weight of all of the embryos of a given species is taken as the level at which the line is drawn. The degree to which these diagrammatic lines correspond to lines calculated through the data is discussed below. An approximation of the standard deviation observed from the above lines for each species is given by SE_y in Table I. *Heterandria formosa* is included as an example of a truly viviparous fish. The other species have previously been considered to be ovoviviparous. *Gambusia holbrookii* should be listed as *Gambusia affinis holbrookii*. *Poecilistes* sp. Peten has been identified by Hubbs as *Poecilistes pleurospilus* from Guatemala.

[4] Measurements on this species were also made in Panama during the summer of 1940. The weight of the fertilized egg and of the larva at parturition are indicated in Figure 1. A more complete discussion of this species is being prepared.

for *Heterandria* is probably legitimate (Scrimshaw, 1944). The broken lines indicate incomplete data. The known oviparous fishes have egg and larva weight greater than those of any of the fishes shown in Figure 1.

In Figures 1–6 no correction has been made for the effect of the fixative on the dry weight of the embryo. As can be seen from Figure 5, no single correction can

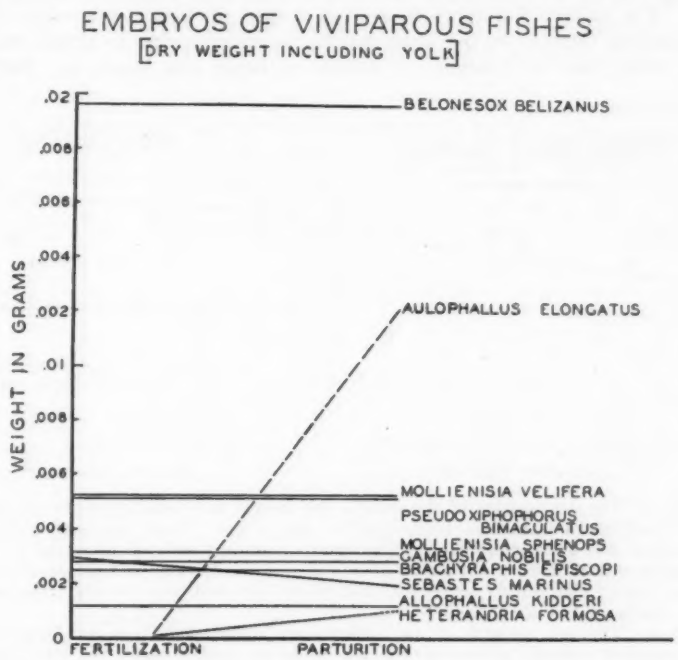

FIGURE 4. Additional species are shown in a manner identical to that described for Figure 3. The scale is enlarged to include species with greater embryonic weights. *Aulophallus* and *Heterandria* represent truly viviparous poeciliids. *Sebastes marinus* is a viviparous perch, a much larger fish than any of the others discussed and not a member of the Poeciliidae. It is included as an example of a truly ovoviviparous fish.

be made for preserved material. Some embryos of *Gambusia affinis holbrookii* preserved since 1878 have changed very little in dry weight when compared with living ones studied in the laboratory. On the other hand, certain material collected in 1919 from the same place as the laboratory stock collected in 1941 has lost at least 70 per cent of its original dry weight. These specimens, however, were fixed in alcohol and preserved in strong alcohol (as high as 90 per cent). The 1878 material was fixed in formalin before it was transferred to the strong alcohol used as a preservative for fish by the Museum of Comparative Zoology. The material obtained from the Michigan Museum of Zoology was uniformly treated, having been fixed in 10 per cent formalin and changed to 70 per cent alcohol for storage.

The dry weight of the embryo in fishes treated in this manner closely approximates that of the living form. The data plotted for *Brachyrhaphis cascajalensis* in Figure 2 illustrate this.

In Figure 4, the *Poecilistes* species has been identified by Hubbs as being a race of *Poecilistes pleurospilus* from Guatemala. The *Poecilistes pleurospilus* listed is laboratory stock obtained from the Everglades Aquatic Nurseries, Tampa, Florida. The marked difference in average weight of these two groups of specimens, assuming them to be the same species, is probably due to actual racial differences rather than to fixation. A similar situation was found for *Mollienisia*

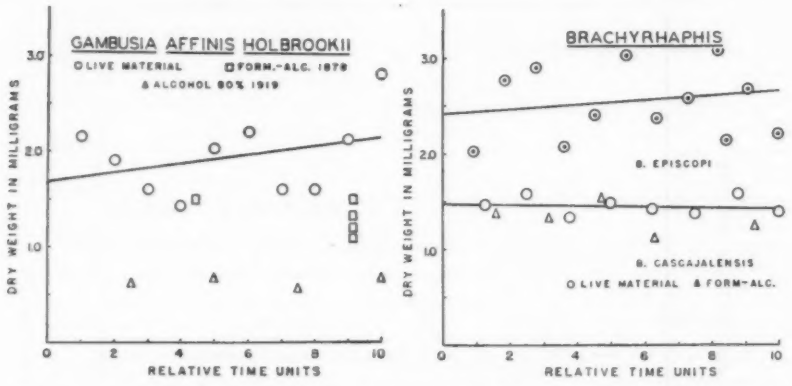

FIGURE 5. The dry weights of the embryo are plotted in a manner similar to Figure 1. The line is calculated from data represented by the circles. These represent embryos dissected out alive and dried immediately. The squares represent material preserved in formalin in 1878 and later transferred to the strong alcohol used in the Museum of Comparative Zoology, Cambridge, Mass. This varied between 75 and 90 per cent and was the sole preservative for specimens represented by the triangles.

FIGURE 6. The dry weights of the embryo are again plotted as in Figure 1. The triangles represent material preserved originally in 10 per cent formalin and later kept in 70 per cent alcohol. When weighed several months later there was excellent agreement with the values for living material studied at the time these were preserved. Most of the preserved material described has been handled in this manner.

sphenops. Two forms which could be distinguished by size, coloration, and mean weight of the embryos were taken from Gatun Lake. The mean weight of the larger form is plotted in Figure 3 as 3.05 mg. The smaller forms showed a very constant embryo weight averaging 1.58 mg. Despite these differences, Hubbs (1942) was able to find no usual taxonomic character or measurement to justify considering the smaller form a separate species.

Since the sperm in these fishes are viable in the ovary for several months, the time required to reach any given stage of development cannot be determined even in laboratory specimens. Therefore, in this study the embryonic weights were ranked in order of stage of development (cf. Scrimshaw, 1944). Since the data show that there is no consistent dry weight change during development, no error was introduced in the tabulation of the raw data. However, the assumption for

graphical and statistical purposes of an equal interval of time between the stages studied means the use of a relative rather than an actual time scale.

In the figures presented, the first stage was taken to represent the weight at fertilization and the last stage the weight at parturition. The time between was arbitrarily divided into ten units. Thus in the case of *Brachyraphis episcopi* where ten stages were studied, each stage in Figure 6 is separated by an arbitrary relative time unit of 1.0. In the case of *Allogambusia tridentiger* represented by fourteen stages (Fig. 2), the ten point scale must be divided into fourteen parts. Thus each stage is separated by 0.71 unit.

Using relative time units as the abscissa and dry weight in milligrams as the ordinate, the raw data were recorded graphically. It was apparent by inspection that the data for each species could be represented by a straight line. Accordingly these were fitted by the method of least squares and were of the form $y = mt + k$. The intercept (k) of the straight line then represents the weight of the egg at fertilization. The slope (m) describes the weight change during development.

TABLE I

Species	N	k	SE_y	m	$m°$
1. *Gambusia affinis holbrookii*	10	1.676	.1127	.0502	2° 52′
2. *Darienichthyes dariensis*	6	1.925	.0584	−.0197	−1° 8′
3. *Poeciliopsis occidentalis*	12	1.923	.0456	.0027	0° 9′
4. *Poeciliopsis infans*	10	.937	.0108	.0046	0° 16′
5. *Aulophallus kidderi*	7	1.204	.0167	−.0040	−0° 13′
6. *Brachyrhaphis episcopi*	11	2.419	.1237	.0263	1° 30′
7. *Mollienisia sphenops*	12	3.045	.1495	.0204	1° 10′
8. *Dextripenis evides*	7	1.700	.0266	−.0350	−2° 0′
9. *Brachyrhaphis cascajalensis*	8	1.481	.0076	−.0055	−0° 19′
10. *Gambusia dovii*	10	1.667	.0479	−.0330	−1° 53′
11. *Allogambusia tridentiger*	14	1.005	.0132	−.0027	−0° 9′
12. *Xiphophorus helleri**	21	1.841	.1602	−.0009	−0° 3′
Total	118			Average .0034	0° 12′

N, number of embryonic stages; K, intercept of development curve in milligrams; SE_y, standard error or estimate of y in milligrams, $SE_y = \sqrt{\frac{\Sigma d^2_y}{N-1}}$; m, slope of development line; $m°$, deviation of development line from horizontal in degrees.

* Calculated from data of Bailey (1933).

As listed in Table I, the mean slope of all of these lines is only .0034. This is equivalent to an angle 0° 12′, an almost imperceptible deviation from a slope of 0. In order that these small deviations may be more readily visualized, the actual angle in degrees equivalent to the calculated slope is also listed in Table I. From inspection of the data, it can be concluded that the random variations from a slope of 0 can be accounted for by biological variation and sampling error. No significant change in the dry weight of the embryo in the intact ovisac can be detected during development.

A further statistical basis for the above conclusions can be obtained by placing the data for the different species in numerically comparable form.[5] This consists of adjusting the data already calculated to an arbitrary standard. The weight at fertilization (k) was made identical for each species and equal to unity (k_r). All other statistics were scaled accordingly by multiplication with the ratio $1/k$. The resulting curves ($y_r = m_r t + k_r$) are seen in Figure 7 and the new relative statistics are listed in Table II.

TABLE II

Species	m_r	$\hat{\sigma}m_r$	$t_{0.00}$	$t_{-.033}$	$P_{0.00}$	$P_{.033}$
1. *Gambusia affinis holbrookii*	.0300	.0213	1.409	2.961	.2	.02
2. *Darienichthyes dariensis*	−.0102	.0124	− .8270	1.841	.5	.15
3. *Poeciliopsis occidentalis*	.0014	.0068	.2032	5.028	.5	.001
4. *Poeciliopsis infans*	.0049	.0037	.7434	7.464	.5	.001
5. *Aulophallus kidderi*	−.0033	.0053	− .6324	5.653	.5	.001
6. *Brachyrhaphis episcopi*	.0109	.0154	.7049	2.845	.5	.02
7. *Mollienisia sphenops*	.0078	.0142	.5505	2.668	.5	.02
8. *Dextripenis evides*	−.0206	.0593	− .3473	.2093	.5	.5
9. *Brachyrhaphis cascajalensis*	−.0037	.0018	−2.044	6.088	.09	.001
10. *Gambusia dovii*	−.0198	.0091	−2.183	1.452	.05	.2
11. *Allogambusia tridentiger*	−.0027	.0035	− .7749	8.627	.5	.001
12. *Xiphophorus helleri**	−.0005	.0190	− .0268	1.709	.5	.1

k_r, common reference intercept, $k_r = 1.000$ (omitted from table, but essential part of data identical for all species); m_r, slope of relative development curve, $m_r = m \cdot 1/k$; $\hat{\sigma}m_r$, unbiased estimate of standard deviation of m_r; $t_{0.00}$, Fisher-t statistic, $t_{0.00} = \dfrac{m_r - 0.00}{\hat{\sigma}m_r}$; $t_{.033}$, Fisher-t statistic, $t_{.033} = \dfrac{m_r - (-.033)}{\hat{\sigma}m_r}$; $P_{0.00}$, probability that the difference in slope between the observed curve and one of zero slope is due to random sampling error; $P_{.033}$, probability that the difference in slope between the observed curve and one of − .033 slope (parturition weight = 2/3 fertilization weight) is due to random sampling error.

With the statistical measures for the 11 species adjusted in this manner, the impressions gained from the raw data can be evaluated. It was pointed out above that these fishes have been considered by most authors to be ovoviviparous. As will be discussed, this would mean that one-third of the nourishment originally available in the egg would be used before parturition for maintenance purposes. If this is so, the slope of a straight line development curve would be − 0.033 and the final dry weight would thus appear as ⅔ of the initial weight.[6]

The probability ($P_{-0.033}$) that the development curve of one of these species represents a chance deviation from a slope of − 0.033 can be determined by using the relation $t_0 = [m_r - (0.033)/m_r]$ and tables of the Fisher-t distribution. Examination of the $P_{-0.033}$ column in Table II shows that for most of the species examined the P is .05 or below. Thus, it is not probable that any of these curves represents a chance variation from a characteristic

[5] The statistical data in Table II have been calculated by Mr. James Rafferty of the School of Medicine and Dentistry, University of Rochester. The statistical treatment of the data has been carried out entirely by him and the text description of the methods employed has been prepared under his direction.

[6] The slope of such a line would be − 0.33 if the units were equal for ordinate and abscissa. Since there are ten relative time units to a common reference weight of unity, the figure for the calculated slope becomes − 0.033.

slope of -0.033. To conclude that this group of curves as a whole shows chance deviation from such a slope is impossible. It should be apparent that the lines in Figure 7 do not represent a group of ovoviviparous species.

However, is the apparent grouping of the development curves about a slope of 0.00 significant? The probability ($P_{0.00}$) that these lines represent chance variations from a characteristic slope of 0.00 can be found in a similar manner. It will be seen from Table II that the probability in most instances is .5 or better that this is the case. Taken as a whole, it is probable that the modal development curve for this group of species is a horizontal line. Thus the conclusion that there is no change in the weight of the embryo in the intact ovisac is substantiated.

The data for certain of the species require further comment. The data for species 2, 10, and 12 do show $P_{-0.033}$ values of $.1 - .2$. Of these, only species 10, *Gambusia dovii*, shows a real deviation from a line of 0 slope (Fig. 7). In the case of species 8, *Dextripenis evides*, with a $P_{-0.033}$ value of .5, there is enough initial variation in the weights that a deviation from a slope of 0.00 or -0.033 is equally probable.

It might also appear from Figure 7 that species 1, *Gambusia affinis holbrookii*, has pro-

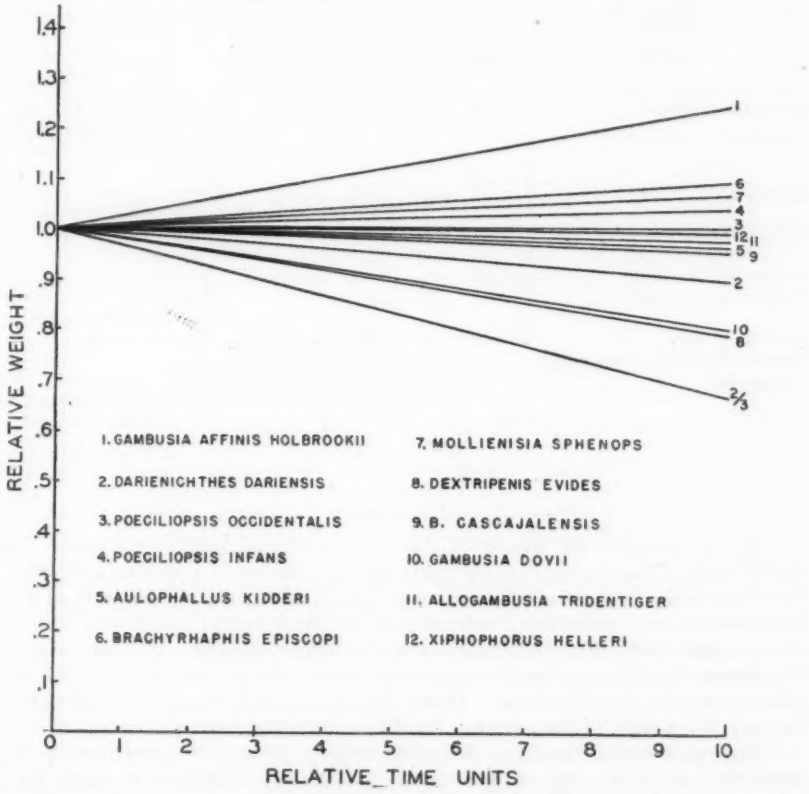

FIGURE 7. The weight data for the different species have been adjusted to an arbitrary standard so that the findings can be compared directly. The weight at fertilization (k) was made identical for each species and equal to unity (kr). All other statistics were scaled accordingly by multiplication with the ratio $1/k$. Thus this new relative weight could be plotted as a function of relative time as used in the previous figures. The $\frac{2}{3}$ line represents the line which the development curve of these species would follow if they were truly ovoviviparous.

gressed further than the other species toward true viviparity. While this possibility cannot be excluded, the P_o value of .20 may be taken to indicate that this deviation from zero slope may be a chance occurrence.

In several species it was not possible to secure enough stages to establish the mean dry weight of the embryo or the change in dry weight with development. The limited data for these species are summarized in Table III. The three females of *Lebistes* studied represented pure inbred laboratory stock and the average embryo weight of all three widely separated stages proved to be 2.3 milligrams. It is probable that most or all of the species in this table would fit into the group represented in Table I if complete data were available.

TABLE III

Dry weight data for additional poeciliid species

Species	Number studied	Average weight (mg.)	a	Adequacy of data
Belonesox belizanus	6	19.7	—	Range of 6.2–15.2 mg. for larva at parturition (6.2 value omitted from average)
Brachyraphis terrabensis	5	2.18	.81	Very narrow stage range and wide variation
Gambusia nobilis nobilis	8	2.8	.80	Only very early stages studied
Lebistes reticulatus	3		.0	Too few stages
Mollienisia vivipara	3	5.4	—	Few stages, very wide variation (3.4–6.3 mg.)
Poecilia vivipara	4	.96	.67	Too few stages
Pseudoxiphophorus bimaculatus taeniatus	5	5.3	—	Early stages only (Excessively low late stage value of 3.2 mg. omitted from average)

DISCUSSION

The variation in the weight of the intact ovisac at various embryonic stages seems to be a characteristic of the particular population of a species sampled. As described above, the standard error of y (SE_y) is actually a composite of errors. Nevertheless, it is primarily a reflection of the physiological and genetic variability to be found within the representatives of the species studied. It is probably valid to compare the inherent genetic variability of one species sample with that of another by means of this statistic. When the SE_y is small enough to minimize overlapping, the weight of the embryos should occasionally prove of taxonomic use.

The experimental data show the weight of the embryo at parturition to be the same as that of the egg at fertilization. Thus, quantitatively at least, the full amount of food material in the egg is converted into the substance of the embryo at the time of hatching. However, it is known that the food requirement of the embryo for maintenance metabolism during development is considerable.

Gray (1928) obtained for the plastic efficiency coefficient (P.E.C.) of the trout a figure of 0.63. This he believes to be a universal value for the synthesis of

protoplasm. This coefficient is based on the dry weight of the yolk that can be accounted for by the dry weight of the fully developed larva. It represents the relative cost in weight units of building the tissues of the embryo. The higher the coefficient, the smaller the amount of burned substance in relation to the amount of nourishment initially available. Table IV summarizes the values for P.E.C. which have been reported. These values suggest that the mother must also contribute an amount of nourishment equal to about one-third the initial weight in the poeciliid fishes studied.

TABLE IV

Plastic efficiency coefficients of development

Species	Author	Coefficient
Trout (*Salmo fario*)	Gray (1926)	.63 embryo
Chick (*Gallus domesticus*)	Murray (1926)	.68 embryo
Frog (*Rana temporaria*)	Fauré-Fremiet & Dragoiu (1923)	.58 embryo
Silkworm (*Bombyx mori*)	Farkas (1903)	.59 embryo
Mould (*Aspergillus niger*)	Terroine & Wurmser (1922)	.59 normal growth
Viviparous perch (*Sebastes marinus*)	Hsiao (unpublished data)	.66 embryo
Trout (*Savelinus fontinalis*)	Gortner (1912)	.74 embryo (heat dried)

The maintenance cost of development is also illustrated by the various measures of the energy required during development. This subject has been well summarized by Needham (1931). Table V shows the values which have been obtained experimentally for one type of energy coefficient, the apparent energetic

TABLE V

Apparent energetic efficiency of embryonic development

Species	Author	Efficiency
Chick (*Gallus domesticus*)	Tangl (1903)	62.9%
Chick (*Gallus domesticus*)	Murray (1926)	67.0
Fundulus (*Fundulus heterolitus*)	Glaser (1912)	52.8*
Frog to hatching (*Rana tempororia*)	Fauré-Fremiet & V. du Streel (1921)	82.0
Frog to end of yolk (*Rana tempororia*)	Fauré-Fremiet & Dragoiu (1923)	51.0

* This value was obtained by assuming that the same energy relationships prevailed for the use of the final half of the yolk as for the initial half. This figure is certainly too low.

efficiency (A.E.E.), which gives the relation between the chemical energy in the fertilized egg and that combusted during development. For example, the figure cited by Tangl for the chick would indicate simply that 37.1 per cent of the original energy stored in the egg was lost by the time the yolk was consumed and the embryo well formed. All of the available evidence indicates that it is legitimate to generalize and to conclude that in the course of embryonic growth of animals developing from a yolked egg, about one-third of the energy initially available is expended for maintenance metabolism.

The evidence that this relationship is also true for viviparous animals is more difficult to obtain. Rubner (1908) derived values, in part theoretical, for the effi-

ciency of seven common mammals (horse, cow, sheep, pig, dog, cat and rabbit). The agreement of these values with the above data is sufficiently close to permit Needham (1931) to conclude that the real energetic efficiency (R.E.E.) of all embryos, mammalian as well as non-mammalian, is about .66. The R.E.E. involves a correction for basal metabolism, but for poikilothermic animals (and embryos such as those of the chick which are essentially cold blooded for the greater part of their prenatal life) it is so close to the A.E.E. that the two can be considered together.

An energy loss of about 34 per cent in the poeciliid fishes studied should be reflected in an appreciable decrease in the total amount of organic matter. The fact that no change in dry weight can be detected points to an exact correspondence between the weight of food used for maintenance and that of the nourishment supplied by the mother. But why should the majority of species in the family Poeciliidae stop at this identical point in the development of viviparity? Some similar factor or factors must be limiting the growth of the embryo to the initial weight of the egg in all of these species.

This might be the case if some essential factor in the yolk were present in a limiting quantity. If this factor could not be supplied by the mother, no amount of energy from her would permit the embryo to grow larger than the initial supply of this limiting substance would allow. Such might be the case if, for example, only simple sugars and certain amino acids were able to cross the placental barrier. Then certain of the essential amino acids, fats, vitamins or other factors might be available to the embryo in restricted quantities. However, the exact correspondence between initial and final weight in nearly all the species is difficult to understand on this basis.

In these fish the nourishment must pass through five to seven tissue layers: the maternal endothelium, maternal connective tissue (theca), maternal epithelium (follicular epithelium), possibly the chorion and vitelline membrane, yolk sac epithelium, embryonic endothelium, and probably some embryonic connective tissue. This situation resembles most closely the epitheliochorial type of placenta found in such animals as the pig. Despite the number of membranes in this type of placenta all of the materials necessary for growth and maintenance pass from the mother to the fetus across this barrier.

In some mammals, the materials most important for the elaboration of embryonic tissues, the amino acids, pass through the placenta against a concentration gradient in a manner suggesting a special mechanism (Needham, 1942). If a completely viviparous fish such as *Heterandria formosa* possessed such a mechanism, the steady gain in weight of its embryos can be understood. If, on the other hand, the fish discussed here lack such a mechanism, the failure of their embryos to gain weight might also be accounted for.

It is also of interest to note that carbohydrate is absent from eggs of oviparous fishes. Needham (1931), in summarizing the available data, expresses the belief that this is true because of the impossibility of supplying an oviparous form with sufficient sugar for embryonic metabolism without vastly increasing the size of the egg. However, there is no reason to believe that carbohydrate would not be used by their embryos in preference to the stores of fat and protein, if it were available. In the case of the mammalian fetus, Windle (1940, page 212) concludes from the available evidence that the fetus of the mammal practices a rigid glycogen

economy at the expense of its mother's dextrose, drawing upon its own liver only in emergencies. It seems reasonable to expect that carbohydrate would also be the most readily available food which the embryo of the viviparous fish can obtain from the mother. Perhaps in the species discussed, carbohydrate from the mother exerts some sparing action on the tissue building materials of the yolk.

The discussion has been carried to this point to suggest something of the direct study which should be made on the functioning of the pseudo-placental barrier in these forms. The controlling mechanisms which are actually operating in the development of the species reported will not be clear until such experiments are carried out.

Summary

In poeciliid fishes the embryos are retained within the ovarian follicles until parturition. When the dry weight of the intact follicle is determined at various stages of development, no significant weight change can be demonstrated in the 18 species studied. This is in marked contrast to the situation in a truly ovoviviparous fish where one-third of the initial weight of the egg is used for maintenance during development. Accordingly, it is concluded that all of the species studied receive nourishment from the mother and hence are not ovoviviparous in the strict sense of the word. Furthermore, they apparently receive only as much nutriment, quantitatively at least, as they require for their maintenance metabolism. Some limiting mechanism must be operating, but its nature is not known. This situation is contrasted with that in *Heterandria formosa* and *Aulophallus elongatus,* members of the family which have developed nearly complete dependence on the mother for nourishment during development.

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THE EFFECT OF TEMPERATURE ON THE WINGS OF DIMORPHOS/DIMORPHOS VESTIGIAL-PENNANT/ VESTIGIAL IN DROSOPHILA MELANOGASTER

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Harnly (1930b) and Stanley (1931) showed a critical temperature for the increase in wing size of the homozygous vestigial flies. This response is direct but not proportional to the rise in temperature. It was found that the introduction of the sex-linked modifier dimorphos into this genotype lowered the critical temperature for wing enlargement 5° C. for the males and 2° C. for the females (Harnly and Harnly, 1935). Again the response was direct but not proportional throughout the viable temperature range. Vestigial-pennant is a reverse mutation from vestigial to a recessive wild type allele. The wings of these flies showed an inverse response to temperature, and a critical point appeared to occur between 28° and 30° (Harnly and Harnly, 1936). It was also found that the heterozygous vestigial-pennant/vestigial wings showed an inverse response to temperature from 16° to 22° and a direct response from 26° through 32°.

These shifts in critical temperatures, the direct or inverse response of wing size to temperature of the homozygotes, and the apparent reversal of dominance with temperature in the heterozygote led to this study of the dimorphos/dimorphos vestigial-pennant/vestigial genotype.

Stocks and Methods

Through a knowledge of the phenotypes produced by dimorphos vestigial, homozygous vestigial-pennant and the heterozygous vestigial/vestigial-pennant at 22° and 25°, it was possible to develop a stock of dimorphos vestigial-pennant. Isogenic stocks of dimorphos vestigial and dimorphos vestigial-pennant were employed in these experiments. Due to the breeding technique (Harnly and Harnly, 1935, 1936) these stocks differ from each other in genotype only at the vestigial locus.

The culture methods, procedure for measurement of wings, etc. were the same as those described in earlier papers of this series (Harnly, 1932, 1936; Harnly and Harnly, 1935). Homozygous dimorphos vestigial-pennant females were mated to homozygous dimorphos vestigial males. Their offspring (dimorphos/dimorphos vestigial-pennant/vestigial) were allowed complete development at 16°, 18°, 20°, 22°, 24°, 26°, 28°, and 30° C. Those F_1 individuals placed at 32° for development were returned to a 26° incubator after the completion of five days (120 hours) of development and there completed their growth and their emergence.

Results

Wing length

The genes used and their symbols are: vestigial (vg), vestigial-pennant (vg^p) and dimorphos (di). The curves for the mean wing length for the di/di vg^p/vg

males and females show an inverse response to temperature increases from 16° to 28°, and are similar in slopes and values to those previously recorded for the wild type (Stanley, 1935) and homozygous vg^p (Harnly and Harnly, 1936). The mean wing lengths in mm. of di/di vg^p/vg males and females are shown in Table I.

TABLE I

Mean wing length in mm., di/di vg/vg^p., 5 trials

°C.	Males				Females			
	Length	±	P.E.	σ	Length	±	P.E.	σ
16°	2.53	±	0.0097	0.1241	2.60	±	0.0089	0.1135
18°	2.49	±	0.0056	0.0724	2.59	±	0.0103	0.1306
20°	2.42	±	0.0055	0.0707	2.53	±	0.0086	0.1101
22°	2.32	±	0.0082	0.1030	2.43	±	0.0119	0.1524
24°	2.25	±	0.0069	0.0897	2.33	±	0.0121	0.1550
26°	2.25	±	0.0066	0.0755	2.35	±	0.0109	0.1257
28°	2.11	±	0.0073	0.0939	2.27	±	0.0094	0.1205
30°	2.15	±	0.0067	0.0859	2.28	±	0.0067	0.0864
32°	2.24	±	0.0081	0.0969	2.32	±	0.0069	0.0886

The di/di vg^p/vg males and females show a gradual decrease in wing length from 16° to 28° beyond which there is a gradual increase in wing length to 32°. Apparently 28° is a critical point since the slope is negative below this temperature and is positive above it. The female wings are consistently longer than those of the males throughout the temperature range. The similarity in slopes and values of the di/di vg^p/vg male and female curves indicates there was no difference in the response of the two sexes in wing length over the range employed.

Wing area

The mean wing areas for di/di vg^p/vg will be found in Table II and are shown

TABLE II

Mean wing area in sq. mm., di/di vg/vg^p., 5 trials

°C.	Males				Females			
	Area	±	P.E.	σ	Area	±	P.E.	σ
16°	1.591	±	0.0090	0.1451	1.327	±	0.0171	0.2161
18°	1.555	±	0.0071	0.0914	1.171	±	0.0171	0.2168
20°	1.482	±	0.0086	0.1106	1.065	±	0.0151	0.1934
22°	1.338	±	0.0086	0.1080	0.964	±	0.0156	0.2006
24°	1.278	±	0.0219	0.2313	1.063	±	0.0200	0.2568
26°	1.267	±	0.0064	0.0730	1.111	±	0.0163	0.1867
28°	1.138	±	0.0080	0.1026	1.219	±	0.0116	0.1492
30°	1.214	±	0.0062	0.0796	1.294	±	0.0065	0.0840
32°	1.291	±	0.0080	0.0957	1.342	±	0.0093	0.1192

in Figures 1 and 2 together with those of Harnly (1930b) and Harnly and Harnly (1935, 1936) for the genotypes indicated.

The male curves for vg^p and di/di vg^p/vg coincide and have a negative slope between 16° and 28°. Between 28° and 32° they diverge sharply, the homozygote having a higher negative value while the heterozygote has a definitely positive slope. Apparently 28° is the critical point for length and area in the males of both genotypes.

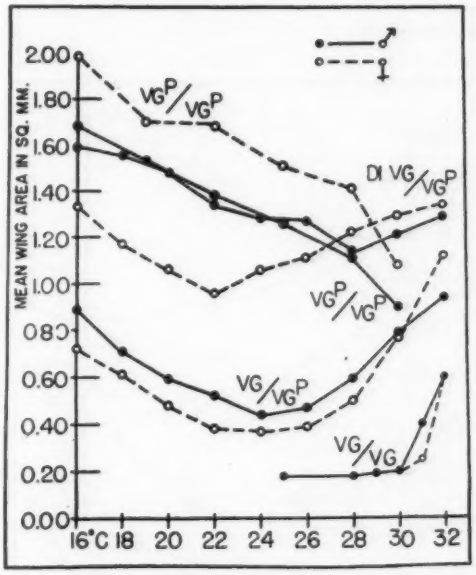

FIGURE 1.

The curve for the di/di vg^p/vg female wing area is U-shaped as is that of vg^p/vg females between 16° and 32°, but their wings are approximately double the area of the vg^p/vg wings. From 16° to 22° there is a gradual decrease and from 22° to 32° there is a steady increase in the area of the di/di vg^p/vg female wings. The reversal in the sign of the slope of the curve at 22° demonstrates that this is the critical temperature for the di/di vg^p/vg females. Between 26° and 28° the female curve crosses the male curve indicating a larger wing area for the females beyond that point up to 32°.

SHIFT IN WING FORM THRESHOLD

The male wing phenotype did not vary significantly from 16° to 32° in the di/di vg^p/vg flies. However, this genotype appears to be responsive to temperature, in that the percentage of perfect wings varied with the temperature. Wings with minor nicks were produced at all temperatures, but perfect wings were only

produced at 16° (19%), 18° (11%), 26° (10%), 28° (7%), 30° (7%), and 32° (28%).

The original homozygous vg^p stock showed only occasional nicks in the distal margins of the wings (Harnly and Harnly, 1936). Repeated back crossing of the vg^p to the inbred vg stock has introduced one or more wing margin genes intensifying this tendency. This resulted in a vg^p stock that regularly showed minor nicks at all temperatures, but produced perfect wings at 16° (Harnly and Harnly, 1936). Due to their origin the homozygous di vg^p and the homozygous di vg stocks presumably carried the same wing margin gene or genes for the production of these minor nicks or notches.

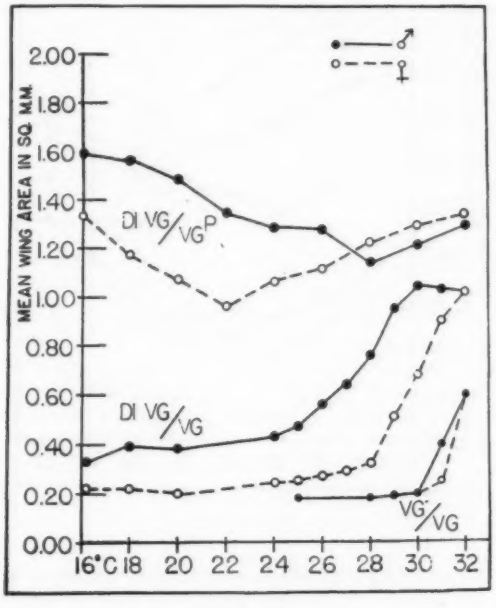

FIGURE 2.

The variation in frequency of nicks through the visible temperature range indicates that the wing-margin gene or genes are thermolabile at some period during the development of the individual. The largest number of perfectly normal wings appear at the temperature extremes, namely 16° and 32°. The smaller percentages of normal wings are found at temperatures close to the upper extreme. This indicates that some genes affecting wing margins are expressed at low temperatures while others find expression at high temperatures. Harnly (1942) obtained parallel results from reciprocal crosses between the two vg^p stocks and between these and a new vg^p stock. The di/di vg^p/vg genotype evidently produces full wings in the males with the possibility of minor variations at the distal end through the action of these wing-margin genes.

The di/di vg^p/vg female wings offer an entirely different picture. From 16° through 26° the phenotype varied around over-sized "antlered" with the occasional appearance of a "strap" wing. At 24° and at 26° phenotypes resembling the vestigial alleles antlered, snipped, carved and notched also appeared; from 28° to 32° the wings were predominantly "notched" to "nicked." Perfect normal wings appear only at 32° and 24 per cent of these wings were normal.

The di/di vg^p/vg female wings at 16° were similar to the male wings of the vg^p/vg described by Harnly and Harnly (1936) at 16°. The introduction of di into the vg^p/vg genotype has lowered the threshold for the wing pattern sequence previously described (Harnly and Harnly, 1935). This results in a pattern grade (antlered) for the female at 16° the equivalent of that first observed between 28°

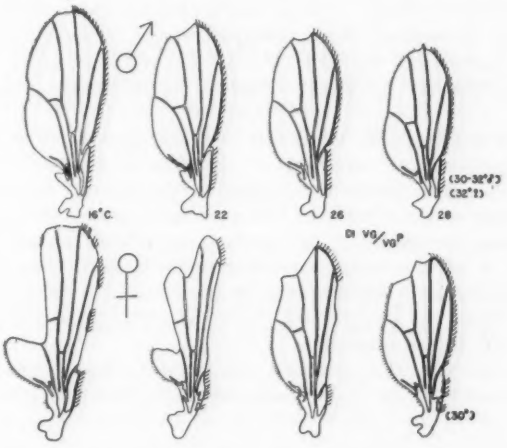

FIGURE 3.

and 30° in the genotype (female vg^p/vg) carrying the normal allele of this sex-linked modifier (Harnly and Harnly, 1936). At these higher temperatures the di/di vg^p/vg females graded phenotypically "notched" to "nicked." The di gene had lowered the pattern threshold some 12° in the females and had lowered it so far in the males that only the full-wing pattern grade was produced throughout the viable temperature range.

SHIFTS IN THE CRITICAL TEMPERATURE

The di mutation arose in an inbred homozygous vg stock and consequently the di vg genotype differed from this inbred vg strain only in the allele present at the di locus. The original vg^p mutant arose in a culture of black vestigial flies. This mutant had been backcrossed for many generations to the inbred vg stock. This produced a vg^p line differing from the inbred vg strain by only the allele at the vg locus. Therefore, these three genotypes differed only by known alleles at the vg and di loci. Consequently the introduction of di into this backcrossed vg^p stock

produced a change in only one locus. The experiments on vg, di vg, vg^p/vg and vg^p (unpublished) have been reported previously (Harnly, 1930, 1936; Harnly and Harnly, 1935, 1936). Including our own experiments on di/di vg^p/vg truly comparable data is now available on five isogenic genotypes.

The di/di vg^p/vg male wings are phenotypically wild type throughout the temperature range employed. However, as mentioned above, "nicks" occasionally appear at the distal margins of the wings. These "nicks" are due to wing-margin genes (Harnly, 1942). The curves for male wing length and wing area coincide with the wild and vg^p curves and demonstrate a similar response to temperature between 16° and 28°. The reversal in the sign of the slope of the di/di vg^p/vg curves between 28° and 32° is markedly contrasted with the further fall of the wild and vg^p curves. This response is due to the combined action of the di and vg genes as will be shown later. The female length curve of di/di vg^p/vg parallels that of the males throughout the temperature range. Evidently under the conditions in these experiments, the threshold for the wild phenotype and temperature reactions of the male and the length response for the female has been reached in this genotype.

The critical temperature is a function of the genotype. The response of vg is direct with temperature and the value of the slope of the curve increases sharply above 30°. The di gene lowers this critical temperature 2° in the homozygous vg female. The slope of the wing area curve of homozygous vg^p females is negative in sign. The wing area curve of the heterozygote vg^p/vg has a negative slope from 16° to 24° and a positive slope from that point to 32°. The area curve of the di/di vg^p/vg females has a negative slope from 16° to 22°, and a positive slope from there to 32°. Again, the introduction of the dimorphos gene has lowered the critical temperature 2°, in the females.

These shifts in the critical temperature from genotype to genotype may be explained as the result of changes in dominance. On that basis the critical temperature (e.g. 24° for vg^p/vg) is the point at which a reversal in dominance occurs between the alleles vg^p and vg in the heterozygote as was suggested earlier by Harnly and Harnly (1936). The data from these experiments with di/di vg^p/vg indicate that there may be two critical temperatures: (1) the reversal of dominance as indicated in the wing area curves; and (2) the phenotype threshold (discussed above), a problem of differentiation as opposed to that of growth in development. It may be that further work will show that both of these are different manifestations of the same phenomenon.

Summary

1. The genotype di/di vg^p/vg was examined at 2° intervals from 16° to 32°.

2. The length of the wings on the males and the females decreased from 16° to 28° and increased from 28° to 32°. The wings of the females were longer than those of the males at all temperatures.

3. The area of the wings of the males decreased from 16° to 28° and then increased to 32°.

4. The critical temperature for both the length and area of the male wings is apparently 28°.

5. The area of the wings of the females decreased from 16° to 22° and increased from 22° to 32°. The resulting U-shaped curve is very similar to that reported

previously for vg^p/vg but the values are approximately double for the di/di vg^p/vg genotype.

6. The critical temperature for the female wing area appears to be at 22° and for wing length at 28°.

7. The di gene lowers the critical temperature for wing area 2° in the vg/vg female and the vg^p/vg female.

8. The critical temperature is a function of the genotype.

9. The males have wild type wings with minor marginal nicks at all temperatures. Wings with perfect margins were produced at 16° (19%), 18° (11%), 26° (10%), 28° (7%), 30° (7%), and 32° (28%). The marginal defects are obviously thermolabile.

10. The wings of the females varied around over-sized "antlered" with the occasional appearance of a "strap" wing from 16° to 26°. Phenotypes resembling the mutant alleles antlered, snipped, carved, and notched appeared at 24° and 26°. The wings were predominantly "notched" to "nicked" from 28° to 32°. Perfect margins were observed on twenty-four per cent of the wings developed at 32°.

11. The di gene lowered the wing pattern threshold some 12° in the females. In the males, this threshold was lowered below the viable range and only normal wings were produced.

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SUBSTRATE-ENZYME ORIENTATION DURING EMBRYONIC DEVELOPMENT [1]

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Introduction

Since Godlewski's (1900) observations it has been known that the rate of respiration increases during the course of amphibian development. Numerous workers (cf. Moog, 1944) have confirmed these results with more accurate measurements. The work of Loeb (1895) and Philips (1940) on Fundulus embryos and Brachet (1934) on amphibians made it evident that a rigid causal dependence of embryogenesis on oxygen consumption does not obtain in all instances. These authors found that a considerable portion of early embryonic development could proceed in the complete absence of oxygen.

Relatively few attempts have been made to analyze experimentally the increase in oxygen consumption during development. Such an increase could presumably involve: (1) an increase in the permeability of the eggs to oxygen during development, (2) synthesis or activation of more enzymes, (3) formation of additional substrate, and (4) reorientation of enzyme and substrate, initially present, but functionally disconnected. This could be accomplished by a spatial separation between the two and the immobilization of a necessary carrier.

The first mechanism is made unplausible by the findings of Parnas and Krasinska (1921), confirmed by Brachet (1934), who found the respiratory rate of amphibian embryos to be, within wide limits, independent of oxygen tension up to neurulation. During this same period a 4 to 5 fold increase in rate of oxygen consumption is realized.

It seemed probable that a systematic comparison of the respiration of brei and intact eggs during development combined with an examination of the cytochrome oxidase content could provide data which would permit a decision amongst the last three hypotheses mentioned. The present paper presents the results obtained from such experiments. They support the hypothesis that control of respiratory rate in developing embryos is effected by a spatial orientation of enzyme and substrate. No detectable increase in the cytochrome oxidase content was observed and the substrate content decreases rather than increases during development.

Materials and Methods

Eggs

The eggs of *Rana pipiens* were obtained after injection of anterior pituitary gland. They were artificially fertilized and after swelling of the jelly were cut up

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into small groups in 10 per cent Ringers solution and kept at 20° C. The stages at which the eggs were selected for measurements were determined according to the schedules of Pollister and Moore (1937) and Shumway (1941). Where desirable intermediate stages were devised with the aid of photographs published in Rugh's (1941) manual.

In preparation for an experiment, the eggs were plucked clean of their jelly with the aid of a pair of fine forceps and filter paper. The denuded eggs were immediately placed in the M/15 phosphate buffer at pH 6.5. The number of eggs used in each flask varied at different stages. The attempt was made to use enough material to give ca. 5 mm. movement of the manometer fluid for each ten minute reading. The eggs were suspended in sufficient fluid so that the total volume was 2 cc. and transferred to the respirometer vessel.

Manometric measurements

All measurements were carried out at 26.2° C. in Barcroft-Warburg Manometric respirometers. The vessels were provided with side arms for substrate additions. The rate of shaking was 100 complete oscillations per minute with an amplitude of 8 cm.

Preparation of brei

After some preliminary experiments the following was adopted as the most satisfactory available method for obtaining breis of the eggs: The correct number of eggs was diluted to 20 cc. with chilled M/15 phosphate buffer and the suspension placed in a Waring blender. This volume is sufficient to almost cover the blades. When for various reasons less volume was used, tilting of the blender was resorted to during its operation. The blender was turned on intermittently until complete destruction of cell structure was attained as determined by direct microscopic observations. Care was taken to keep the temperature of the fluid below 25° C. during the process. Aliquots of the cell-free suspension were then dispensed to the vessels.

It may be noted that attempts to prepare cell-free breis by grinding with purified sand invariably led to considerable inactivation of the respiratory enzymes as evidenced by the consistently lower rates of oxygen consumption of such preparations as compared with those made with the blender. We had no success with the violent shaking procedure described by Brachet (1934). Fewer than 20 per cent of the embryos were disrupted by this method which in Brachet's hands yielded almost 100 per cent cytolysis. This difference may be attributed to a relatively greater sensitivity of the European species (*Rana temporaria*) to mechanical disruption.

The respiration of the breis starts to decline after about 60 minutes. Consequently rates were determined in the earlier constant rate portion. All values reported were obtained by measuring the slope of the straight line from 0 to 60 minutes.

Cytochrome oxidase

Cytochrome oxidase was measured following the precautions noted by Stotz (1939). In excess of 10^{-4} mM. per cc. of cytochrome C prepared by the method

of Keilin and Hartree (1937) from beef heart was included with each suspension in measuring cytochrome oxidase activity. As substrates, paraphenylenediamine (ppd) hydroquinone and adrenaline were employed in concentrations of 1/50th molar. In the case of the latter two, a correction for autooxidation was necessary. This was done by including one flask in which all reagents were used as before except that the cell extract was placed in boiling water for 5 minutes. The readings so obtained were subtracted from those of the other flasks. Such corrected readings did not differ from those obtained with paraphenylenediamine which has a negligible rate of autooxidation. Care was taken to keep the tissue content per flask below levels which would yield respiratory rates exceeding 600 cu. mm. per hour when substrate was added.

Within 30 minutes of the addition of substrate from the side arm, a very sharp drop in the rate of oxygen consumption is observed. Five minute interval readings were taken after the introduction of substrate and the calculation of cytochrome oxidate activity is based on the slope of the straight obtained by plotting the oxygen consumption against time in the 10–20 minute period following the addition of substrate. All the cytochrome oxidase activity measurements on the breis were performed within 60 minutes of their preparation.

Experimental Results

Using the methods described, a detailed comparison was made of the respiratory rates of breis and whole eggs during the course of development. The results obtained are summarized by Figure 1.[3] The general exponential nature of the curve describing the variation of respiratory rates of intact embryos during development agrees with those recently published by previous authors (Atlas, 1938; and Moog, 1944). An exception may be noted in the data reported by Barnes (1944) in which a linear rather than exponential rate of rise is observed between stages 12 and 17. Barnes does not discuss this discrepancy between her results and those published earlier by Atlas.

It may be noted in passing that in agreement with Atlas (1938) and Moog (1944) the curve for intact cells in Figure 1 does not rise continuously. A break occurs between stages 12 and 13, a little later in development than the discontinuities observed by the other authors.

The respiration of the brei is high from the very earliest stages of development and remains so well into stage 13 which corresponds to the onset of neurulation. Beyond this stage however it starts to fall, remaining however above the rate of intact eggs in corresponding stages until about stage 17. Beyond stage 18 the respiration rates of the breis are definitely below those of the corresponding embryos. These results confirm the observations of Brachet (1934) who noted that cytolysis of the eggs of *Rana temporaria* during early embryogenesis led to marked increases in the rates of oxygen consumption whereas later in development decreases always followed cytolysis.

It is quite apparent from the respiratory rates of the breis that the eggs contain from the very onset of development sufficient enzymes and oxidizable substrate to support a much higher rate of oxygen uptake than the intact embryos actually do.

[3] The authors are deeply indebted to Mrs. Helen Spiegelman for her able assistance in making the many calculations.

From this result alone it would seem unnecessary to postulate the formation of additional enzyme or substrate to account for the rising respiratory rate during development. The early high rates of the breis can be explained by assuming that the destruction of the cellular structure permits a freer contact of substrate with enzyme than occurs in the normal embryo. The fall in respiration of the brei observed beyond stage 13 can also be explained on the same basis. With the passage of time more and more substrate is consumed resulting in a depression of the brei respiratory rate due to substrate, or possibly carrier, dilution.

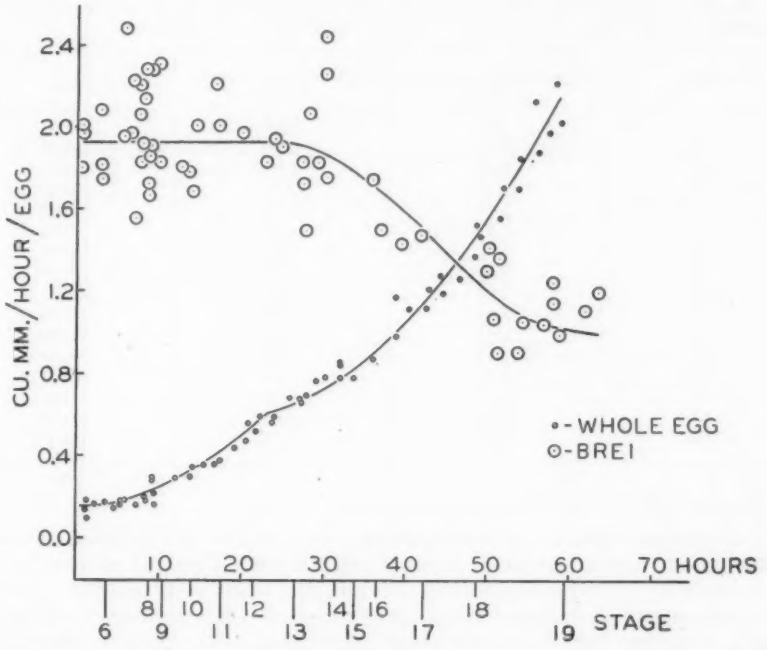

FIGURE 1. Comparison of respiration rates of brei and whole embryo (egg) during the course of development.

Response to cytochrome C

While it was not expected that externally placed carriers could penetrate into the cells, experiments were performed in which very heavy concentrations of cytochrome C, up to 2×10^{-3} mM. per cc., were placed in the external media in which embryos in early stages were suspended. No effect on respiratory rate could be detected.

Since the fall in brei respiration beyond stage 13 might possibly be due to a decrease through loss or inactivation of cytochrome C, this substance was added to breis prepared at various stages of development. Control readings were taken for

approximately ½ hour before transferring sufficient cytochrome C from the side arms to the main compartment to give a concentration of at least 10^{-4} mM. per cc. This concentration was chosen since it gave maximum rates when substrates of the cytochrome oxidase system were also added. The results are summarized in Table I. It is evident from this table that no significant changes occur on adding cytochrome C. Thus brei respiration is not limited by the content of this factor and the fall in rate beyond stage 13 cannot be ascribed to variations in its concentration.

TABLE I

The effect on respiration rate of adding cytochrome C to brei prepared at different stages of development. All values are based on the cu. mm. of O_2 consumed/egg/hour.

Stage	Respiration before addition of cyt. C	Respiration after addition of cyt. C	Change
3	1.91	1.90	−0.01
5	2.20	2.10	−0.10
6	1.85	1.90	−0.05
7	2.04	2.04	0.00
8	2.13	2.21	+0.08
9	1.92	1.92	0.00
10	1.81	1.87	+0.06
11	2.00	2.00	0.00
12	1.92	1.81	−0.11
13	2.06	2.00	+0.06
14	1.62	1.62	0.00
15	1.72	1.70	−0.02
16	1.41	1.30	−0.11
17	1.22	1.22	0.00
18	0.93	1.02	+0.09
19	1.15	1.20	+0.05

Response to addition of cytochrome oxidase substrates

Paraphenylenediamine or hydroquinone was added in the presence of excess cytochrome C to breis prepared from different stages. Figure 2 summarizes the data obtained. The respiration level following the addition of substrate is, under the conditions of these experiments, proportional to the cytochrome oxidase activity. It is clear from the results that this activity is, as far as can be determined, constant throughout development. Consequently neither the rise in the respiratory rates of the intact embryos nor the fall in the respiratory rates of the breis observed in the later stages can be ascribed to variations in the content of this enzyme. The data obtained with added substrates are consistent with the view that the burst of respiration following cytolysis is due to the breakdown of controls, exerted by geometrical constraint, on existent substrates and enzymes.

It is worthy of note that the addition of the cytochrome oxidase substrates raises the respiration at all stages considerably above that attained by the brei alone. This might indicate that even in the early stages all the available active centers of the enzyme are not completely saturated by endogenous substrate when a brei is prepared.

The results of adding the cytochrome oxidase substrates with or without excess cytochrome C to intact eggs at different developmental stages are summarized in

Table II. For purposes of comparison the same table includes data on the rates of breis following similar additions. For the most part these breis were prepared from the same groups from which individuals were used for the intact embryo experiments.

It is clear that in comparison with the marked increases obtained with the breis, the addition of substrate has relatively little effect on the respiration of intact embryos. No consistent strong increase is obtained at any time from stage 3 up to and including stage 19.

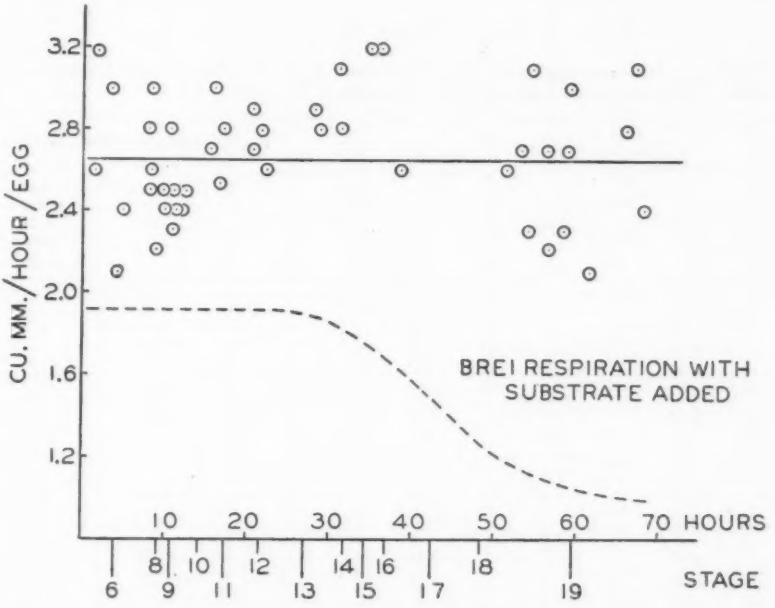

FIGURE 2. The effect on respiration rate of adding substrates of cytochrome oxidase to brei during development. The dotted line represents the curve taken from Figure 1. Single points represent individual experiments.

It is impossible to explain these results on an inability of these substances to penetrate the cells since all of the agents are effective in stopping further development and cause a severe bleaching of the melanin pigment. Both of these facts indicate with some certainty that substrates do penetrate the cells. Thus a simple lack of substrate does not limit the rate of respiration of the intact cells. Carrier immobilization would appear to be the most plausible explanation, although it is conceivable that endogenous substrate somehow forms a protective shell around active centers.

EXPERIMENTS WITH INHIBITORS

Assuming that the respiration is largely mediated through the cytochrome oxidase system, it was of some interest to examine the action of inhibitors of cyto-

chrome oxidase. According to Keilin and Hartree (1939) both cyanide and azide are effective against this enzyme.

Response of brei and intact embryos to NaCN

In these experiments 0.001 M NaCN was used since it was found by preliminary experiments that higher concentrations did not give significantly greater depressions. The usual precautions suggested by Krebs (1935) were observed and appropriate mixtures of alkali and cyanide were used in the center wells. The results

TABLE II

Respiration (cu. mm./egg/hour) of brei and whole eggs on addition of cytochrome oxidase substrate. Substrate concentration M/50; cytochrome C 10^{-4} mM.

Stage	Whole egg	Brei	Whole egg substrate	Brei substrate	Change on adding substrate	
					Whole egg	Brei
3	0.21	1.6	0.20	3.2	−0.01	+1.6
	—	1.7	—	2.6	—	+0.9
	—	2.1	—	3.0	—	+0.9
4	0.21	—	0.20	—	−0.01	—
	0.20	—	0.20	—	0.00	—
	0.25	—	0.21	—	−0.04	—
5	0.17	1.8	0.26	2.1	+0.09	+0.3
	0.15	—	0.16	—	+0.01	—
	—	1.8	—	2.4	—	+0.4
7	—	2.2	—	2.2	—	0.0
	0.20	2.2	0.40	3.0	+0.20	+0.8
	0.15	2.2	0.20	2.5	+0.05	+0.3
	—	2.2	—	2.6	—	+0.4
	0.23	—	0.24	—	+0.01	—
	0.26	1.9	0.21	3.0	−0.05	+1.1
8	—	1.7	—	2.4	—	+0.7
	0.27	2.3	0.29	2.3	+0.02	0.0
	0.30	2.4	0.31	2.4	+0.01	0.0
8^{3+}	0.18	1.7	0.26	2.8	+0.08	+1.1
	0.20	2.1	0.22	2.4	+0.02	+0.3
	0.20	—	0.23	—	+0.03	—
8^{5+}	—	1.7	—	2.5	—	+0.8
8^{9+}	—	2.3	—	2.5	—	+0.2
8^{12+}	0.35	—	0.32	—	−0.03	—
	—	1.9	—	2.5	—	+0.6
10	0.40	2.1	0.60	3.0	+0.20	+0.9
	0.34	2.2	0.30	2.8	−0.06	+0.6
	—	2.0	—	2.7	—	+0.7

TABLE II—*Continued*

Stage	Whole egg	Brei	Whole egg substrate	Brei substrate	Change on adding substrate	
					Whole egg	Brei
12	0.45	1.9	0.50	2.6	+0.05	+0.7
	0.58	1.7	0.58	2.8	0.00	+1.1
	—	1.8	—	2.7	—	+0.9
	0.50	2.2	0.53	2.9	+0.03	+0.7
12^{a+}	0.57	—	0.70	—	+0.13	—
	0.60	—	0.56	—	+0.06	—
13	0.67	2.4	0.67	3.1	0.00	+0.7
	0.64	2.6	0.64	2.9	0.00	+0.3
	0.65	2.9	0.65	2.8	0.00	−0.1
13^{a+}	0.78	—	0.78	—	0.00	—
	0.75	—	0.63	—	−0.12	—
14	0.96	1.3	0.87	2.8	−0.09	+1.5
	—	1.6	—	3.1	—	+1.5
16^{1+}	1.1	1.3	0.83	3.2	−0.18	+1.9
	—	1.2	—	3.2	—	+2.0
	1.2	—	0.94	—	−0.26	—
16^{2+}	—	1.3	—	2.5	—	+1.2
18^{1+}	—	0.78	—	2.2	—	+2.1
18^{2+}	1.7	1.4	1.8	2.6	+0.1	+1.2
18^{3+}	2.1	1.0	2.2	3.1	+0.1	+2.1
	2.1	0.90	2.6	2.9	+0.5	+2.0
18^{5+}	1.6	0.98	1.2	2.3	−0.4	+1.3
	—	0.89	—	2.7	—	+1.8
18^{a+}	—	0.89	—	2.7	—	+1.4
18^{12+}	1.9	0.97	1.6	2.1	−0.3	+1.1
	2.1	0.90	1.8	2.7	−0.3	+1.8
	—	1.00	—	2.3	—	+1.3
19	2.4	1.0	2.8	2.4	+0.4	+1.4
	2.6	0.97	2.0	2.8	−0.6	+1.83
	—	0.85	—	3.1	—	+2.25

obtained are summarized in Figure 3. The data show that a strong inhibition of whole egg respiration is obtained at all stages. The respiration of the brei, while not diminished to levels quite as low, is also strongly depressed. Figure 4 gives the trend of cyanide sensitivity in terms of per cent inhibition and is based on calculations of average values from Figures 1 and 3. The fall in sensitivity of the brei in

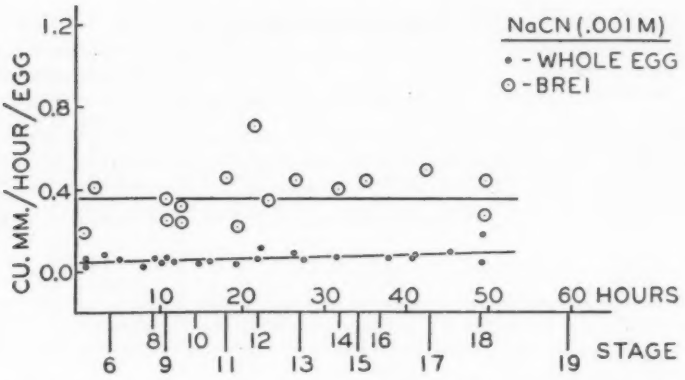

FIGURE 3. Respiratory rate in the presence of cyanide of breis and intact embryos during development.

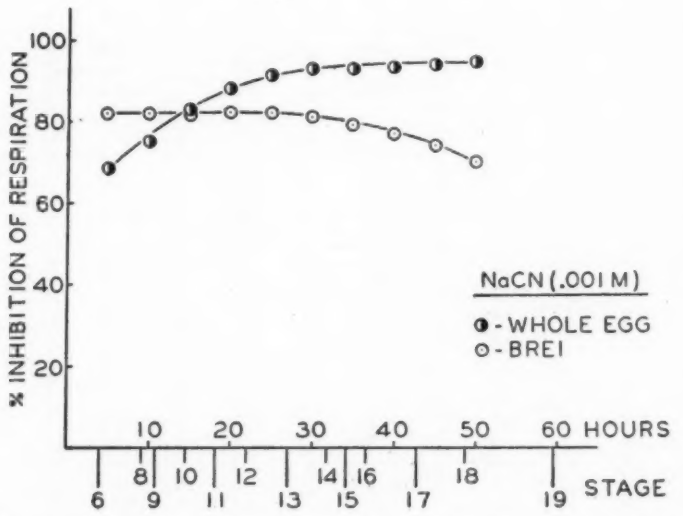

FIGURE 4. Cyanide sensitivity of intact embryo and brei respiration during development.

the later stages is a numerical consequence of the diminishing normal rate during this period which has been attributed to substrate limitations.

These data show that a major portion of the respiration of both brei and intact eggs is mediated via a cyanide sensitive system and that the increments in respiratory rate observed with development are due to the gradual augmentation in the activity of this cyanide sensitive system.

Response of intact embryos and brei to sodium azide

The same type of experiments as were carried out with NaCN were also performed with sodium azide at pH 6.7. The concentration selected was 0.005 M since higher concentrations up to and including 0.1 M did not result in more pronounced effect. The respiratory rates of intact embryos and corresponding breis obtained in the presence of azide are given in Figure 5. The percentage inhibitions at the various developmental stages calculated on the basis of averages from Figures 1 and 5 are shown in Figure 6.

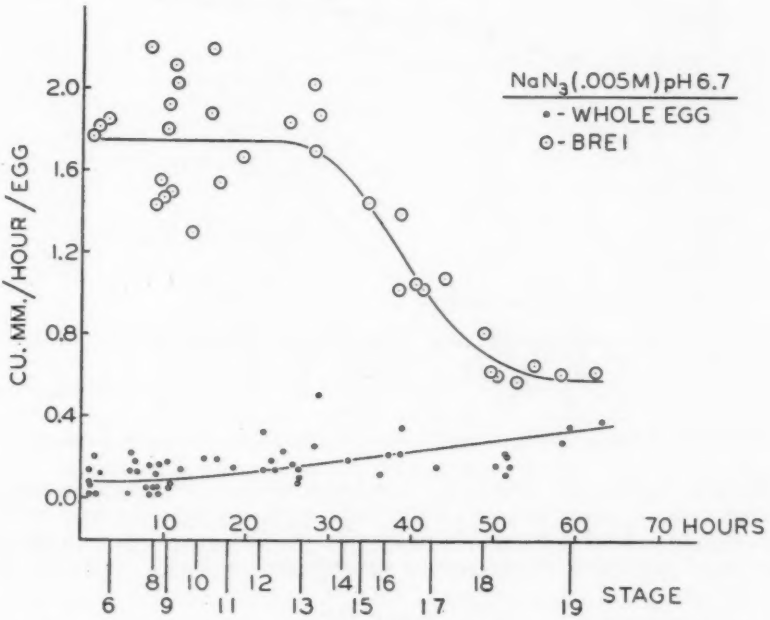

FIGURE 5. Respiratory rates in the presence of azide of breis and intact embryos during development.

In general (Figs. 5 and 6) the behavior of intact embryos towards azide is not very different from that with cyanide. However the residual azide insensitive respiration is consistently greater than its cyanide sensitive counterpart throughout development. Furthermore, unlike the cyanide insensitive respiration which remains constant, the absolute value of the azide insensitive respiration rises during development.

The actions of cyanide and azide on brei are strikingly different. Up until stage 13, 0.005 M azide can decrease the respiration of brei by only 10 per cent. It should be emphasized that this relative ineffectiveness of azide as a respiratory inhibitor against brei is not confined to this concentration only. As may be seen from column 2 of Table III it is true for concentrations as high as 0.1 M.

While Keilin and Hartree (1939) came to the conclusion that azide was virtually equivalent to cyanide in inhibiting the cytochrome oxidase system, recent experiments (Winzler, 1943; Stannard, 1939; Armstrong and Fischer, 1940) show that this conclusion is not universally applicable.

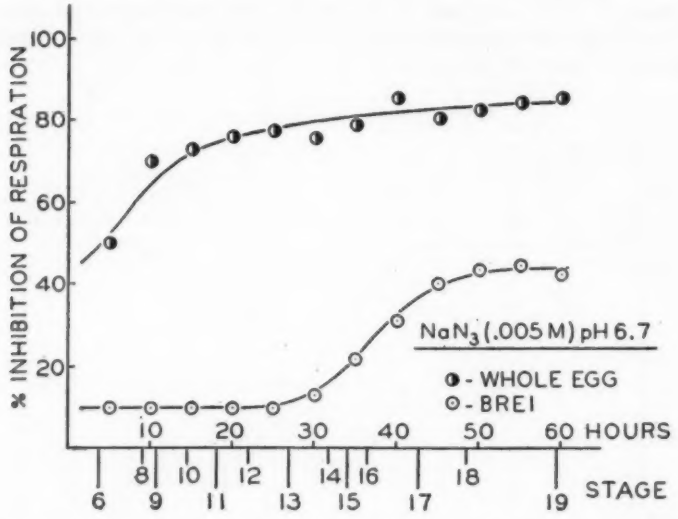

FIGURE 6. Azide sensitivity of intact embryo and brei respiration during development.

To test whether azide attacks the cytochrome oxidase in the breis in the concentrations employed as well as at higher ones, breis were prepared from embryos in stage 9 and placed in the main compartment of Warburg vessels with adequate amounts of cytochrome C and various concentrations of NaN_3. Sufficient para-

TABLE III

Effect of various concentrations of NaN_3 on O_2 consumption of brei when paraphenylenediamine is added. Rate is cu. mm./egg/hour. All eggs in stage 9.

M conc. of azide	Rate before substrate addition	Rate after substrate addition	Δ
0.000	1.91	2.51	+0.60
0.005	1.70	2.29	+0.59
0.01	1.80	2.50	+0.70
0.05	1.63	1.75	+0.13
0.10	1.65	1.67	+0.02

phenylenediamine was placed in the side arms to yield a final concentration of 1/50th molar. After an adequate number of readings were taken to establish the rate without substrate, the substrate was introduced and the resulting respiration followed.

The results are given in Table III. Concentrations up to and including 0.01 M are unable to prevent the rise which occurs on adding substrate. The two higher concentrations tested, 0.05 and 0.1 molar, although ineffective against the respiration of the endogenous substrate, were capable of preventing the burst which normally follows the addition of paraphenylenediamine.

Similar experiments were carried out with cyanide (Table IV). Cyanide not only depresses the brei respiration to levels previously noted but in addition prevents any rise when the paraphenylenediamine is added.

TABLE IV

Effect of various concentrations of NaCN on O_2 consumption of brei when paraphenylenediamine is added. Rate is cu./mm./egg/hour. All eggs stage 9.

M conc. of NaCN	Substrate rate before addition	Rate after substrate addition	Δ
0.000	2.14	2.86	+0.72
0.001	0.51	0.51	0.00
0.002	0.32	0.38	+0.06
0.003	0.24	0.24	0.00
0.005	0.44	0.54	+0.10

These experiments with cyanide establish with some degree of certainty that the normal brei respiration is mediated by the Warburg-Keilin system. At least three important criteria for this assertion are satisfied: (1) The system exists in the brei. (2) The response to cytochrome oxidase substrate is cyanide sensitive. (3) The respiration of endogenous substrate is also cyanide sensitive. It is therefore necessary to assume that insensitivity of the brei respiration to azide is due to the latter's inability to combine with the cytochrome oxidase in the brei. It is conceivable that azide cannot combine readily with the cytochrome oxidase when the other enzymatic components and substrates of the cell are present in the extract.

It is certainly reasonable to expect that the sensitivity of a given enzyme system to a particular inhibitor will depend on the kind and amount of the substrate with which it is competing for active centers. It is therefore not surprising that cytochrome oxidase should be more sensitive to azide when in comparative isolation. That some such competitive mechanism is working may be seen from an analysis of the data in Table III and in particular from the fact that the two highest concentrations employed, while leaving the respiration of endogenous substrate relatively untouched, effectively suppress the increased oxidation when paraphenylenediamine is added. These findings would be expected if at the higher concentrations of azide the inhibitor could compete successfully for an active site on the enzyme surface with a molecule of paraphenylenediamine but not with a molecule of endogenous substrate. Further, it will be recalled (see Fig. 6) that azide sensitivity of the brei respiration starts to rise beyond stage 13 when the normal rate starts to fall due to substrate limitations. This finding would also be expected, since with substrate concentrate falling the ratio of azide to endogenous substrate rises and the azide can then start to compete successfully for active centers.

While the above interpretation fits all the data obtained on the relative azide insensitivity of brei it leaves unexplained the effective inhibitory action on intact embryos shown in Figure 5. If we interpret this inhibition as a reaction between the Warburg-Keilin system and azide we would necessarily conclude that this agent can combine with cytochrome oxidase in intact eggs but not in cell extracts. A plausible reason for this difference may be seen by focussing attention on substrate availability in the two situations. Here again we probably have a situation analogous to the increasing azide sensitivity of brei when the rate is falling i.e., less severe competition by endogenous substrate molecules for active centers as their concentration decreases. It is undoubtedly true that in the intact eggs the number of substrate molecules getting to the enzyme surface is severely regulated, permitting the azide molecules to compete successfully.

It is possible to explain the different responses of brei and whole egg to azide by assuming that in neither case can the azide combine with cytochrome oxidase in the concentrations employed. The capacity of azide to inhibit synthetic processes has been demonstrated for certain systems (Winzler et al., 1944; Moog and Spiegelman, 1942). Most important for the present discussion is the finding by Spiegelman and Moog (1945) that azide completely inhibits amphibian development at all stages including those between fertilization and gastrulation. From this point of view then, azide may depress the respiratory rates of intact eggs indirectly because it inhibits synthetic activities leading to substrate availability for enzyme action.

Discussion

The data presented find their most plausible interpretations in terms of structure orientation and the consequence of its destruction. The comparison of brei and intact embryos in the course of development as well as the determination of cytochrome oxidase content ruled heavily against enzyme or substrate synthesis as explanations for the rising respiratory rate during development. This emphasis on the internal geometry of enzyme and substrate and its variation as determining factors is a point of view that is becoming increasingly popular. Recent reviews by Korr (1939) and Commoner (1942) have stressed it as a criterion for evaluating data obtained from in vitro experiments. Both Runnstrom (1930) and Korr (1937) have discussed the sudden activation of the Warburg-Keilin system on fertilization of Arbacia eggs in terms of the relative positions of enzyme and substrate. Ballentine (1940a, b) who studied the dehydrogenase activity in the same material suggested similar considerations for these enzyme systems. It seems inescapable that more refined interpretations of physiological processes must consider not only what enzymes and substrates exist in the cell, but also where they exist. The present investigation which explains rising respiratory rate in terms of reorientation of existent components indicates that this is equally true for embryonic processes.

Summary

The respiratory rates of whole embryos and cell-free breis were determined at various stages of development. During early stages, when the respiration of whole

embryos is low, brei respiratory rate is high, the situation being reversed beyond about stage 17.

Cytochrome oxidase remains uniformly high throughout development as shown by the effects of adding substrate to breis. Externally applied cytochrome oxidase substrates have little effect on intact embryos although the agents are shown to penetrate. Cytochrome C is shown not to be a limiting factor in brei respiration.

Cyanide depresses the respiration of both breis and embryos. Azide has little effect on respiration of breis but is very effective in depressing oxygen consumption and development of intact embryos.

The results are discussed in terms of spatial orientation of enzymes and substrates.

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PINNOTHERES OSTREUM, PARASITIC ON THE AMERICAN OYSTER, OSTREA (GRYPHAEA) VIRGINICA[1]

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Introduction

The oyster crab, *Pinnotheres ostreum*, was first described by Say in 1817. It is an organism known for many years to oystermen and biologists alike although its habits and life history are but imperfectly known. In her monograph on the grapsoid crabs Rathbun in 1917 defined the adult characters of the genus and compared the numerous species which had been collected from many parts of the world in association with various mollusca, tunicates, annelids and sea urchins. This association has regularly been considered commensalism by most investigators. In the case of *P. ostreum*, however, the relationship is not clear for in 1892, Bashford Dean wrote that the crab was evidently annoying to the oyster for the palps sometimes show thickened out-growths or are malformed and stunted in size. Orton, in 1921, presented clear evidence in the case of the pea crab, *P. pisum*, from the mantle cavity of the mussel, *Mytilus edulis*, that the activity of the crab was definitely parasitic in nature. Ryder, on the other hand, supposed that the crab was of value to the oyster since the latter was said to feed on the clusters of bell-animalcules (*Zoothamnium* sp.) which are attached to the crab's shell. A consideration of the crab's stomach contents, however, showed that its food consists in great part of the minute organisms sought by the host in addition to small crustaceans not normally the oyster's prey.

A sudden increase in the numbers of *P. ostreum* in Delaware Bay in 1941 associated with definite erosions of the gills of the oyster and producing, or at least contributing to, the death of many oysters stimulated a re-examination of the crab. The data obtained (abstracted recently, Stauber, 1942) are the subject of this paper.

Life History

Atkins (1926) has described the post-planktonic stages in the developmental cycle of *P. pisum* from *Mytilus edulis* in British waters. The situation with *P. ostreum* is so similar that, except for morphological details, it seems to be the same. The invasive stages of the oyster crab are small, hard, flat crabs with a carapace 1.4–3.4 mm. wide (Figs. 1, 3, and 23). The sexes are separate and indistinguishable except for the number of pleopods and the genital apertures. Apparently copulation takes place after invasion of the oyster and perhaps is necessary, as Atkins suggests, in order for further development of the female crab to take place. In *P. pisum* she

[1] The author wishes to express his deep appreciation for the many ways in which Dr. Thurlow C. Nelson aided and inspired this work.

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found that in all other stages of the female crabs the spermathecae contained some spermatozoa. Occasionally only one spermatheca was full but more often both were distended with sperms. Except for a few males similar in external appearance to the second stage females no other stage of the male *P. ostreum* is known and the male is believed to be free-swimming like the invasive first stage female.

The second stage female differs markedly from the first stage being rounder in form, grey in color with prominent chromatophores, with a softer carapace and slenderer legs. In size, however, it measures only slightly larger (0.9–3.1 mm.).

The third stage female is still larger (2.6–4.4 mm. wide) but aside from this is quite like the second stage in color, shape and appearance. Higher development of the pleopods differentiates it from the latter.

The fourth stage female is pale yellow in color and is again larger (3.6–8.9 mm. wide).

The adult female, or fifth stage crab, ranges in size from 6–14.9 mm. and is the form of *P. ostreum* commonly known. Indeed, except for this stage, only a few males and none of the other female stages have ever been recorded. Several moults of the fifth stage female crabs are possible and there is some overlapping of sizes of the crabs in various stages due to time of invasion, food supply, size of the oyster invaded, etc. No data are available on the rate of growth or time lapse involved between the various female stages. Only moults of fifth stage females and one moult of a third stage female into the fourth stage have been obtained in the laboratory. In one dredged oyster a fifth stage female and a moulted shell were obtained within the same bivalve.

Ovigerous females are seen in the summer and autumn of the year and Atkins believes that *P. pisum* may become adult in a single year. There is reason to believe that such is the case with *P. ostreum* also. Judging by the size of the mature female crab, with reference to the male copulatory pleopods, it seems unlikely that a second copulation takes place. Therefore, if the large crabs live more than one year, which is quite probable, they must have received sufficient sperm in the one copulation to last for several batches of eggs or become capable of producing only infertile eggs. Orton and also Atkins found the majority of large females of *P. pisum* to have full spermathecae but an occasional large female with empty spermathecae led the latter to postulate that copulation may occur more than once.

After a time, the length of which is still unknown, the developing embryos hatch from the eggs attached to the pleopods of the mother producing a zooea which has been described for *P. ostreum* by Birge (1882) and again by Hyman (1924). In our own experience an ovigerous female was obtained from an oyster dredged as late as October 19, 1942. The embryos then were almost ready to hatch and showed a pulsating heart, a fully formed abdomen, eyes, a digestive gland, and appendages. Hatching began four days later and large numbers of the first zooea were available for study.

Subsequent planktonic stages of this crab are still unknown but judging by Lebour's (1928) criteria (the relatively primitive condition of the first zooea) three or more zooeal stages are probably present and possibly also a megalopa. Eventually, however, the free-swimming invasive male and female crabs are formed completing the cycle.

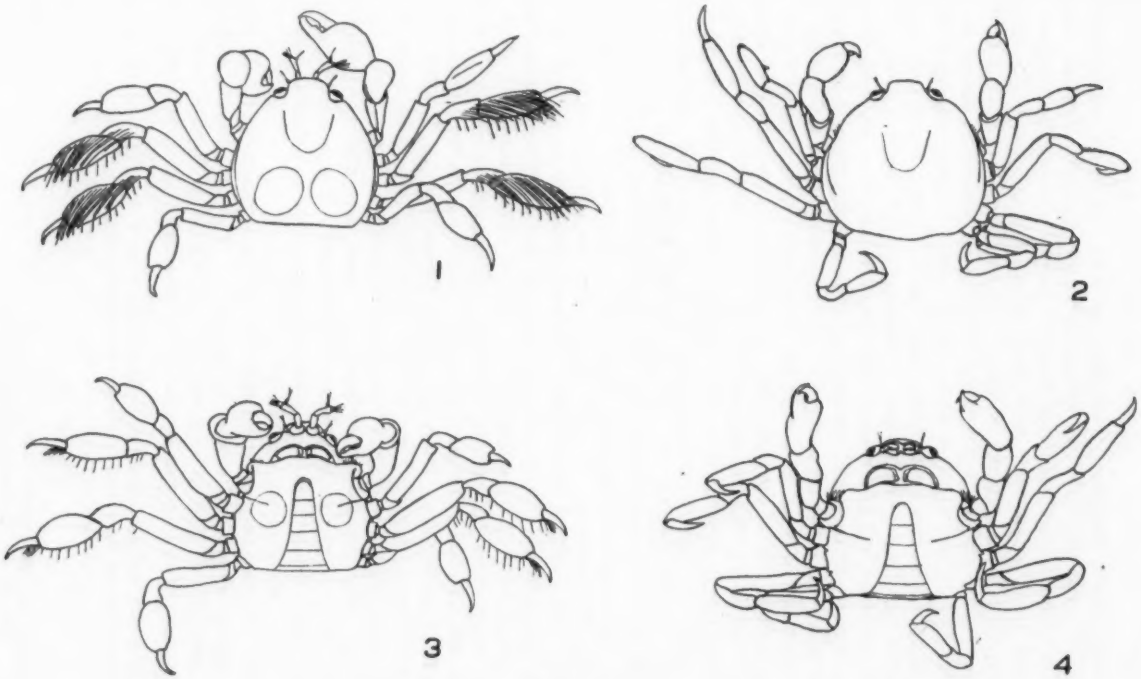

PLATE I

First and second stage female *Pinnotheres ostreum*. × 10

FIGURE 1. Dorsal view of Stage I female.
FIGURE 2. Dorsal view of Stage II female.
FIGURE 3. Ventral view of Stage I female.
FIGURE 4. Ventral view of Stage II female.

Morphology of the Various Stages of the Crabs

The male

In contrast to *P. pisum*, the male *P. ostreum* has been considered an unusual find, Rathbun (1917) reporting only two specimens in the National Museum collection. In size it is much more restricted than the male *P. pisum*, the carapace usually being from 1.5–3.4 mm. wide with sizes below 1.6 and above 2.5 mm. being rarely seen. The mean carapace width of 144 specimens was 2.13 ± 0.19 mm. Furthermore, unlike all but the first stage female from which it is almost undistinguishable, its shell is well fortified with calcium salts probably making it as hard for its size as species of free-living crabs. In color it is dark brown with two large distinct almost circular pale white spots, visible both on carapace and on the sternum. On the posterior half of the dorsal side they are situated in the branchial regions. On the ventral side they flank the groove which contains the abdomen and are situated at the level of and mediad to the first pair of pereiopods or walking legs. In a male, whose carapace was 2.0 mm. wide the dorsal spots were roughly 0.6 by 0.5 mm. On the ventral side of the same crab the pale areas were only 0.45 mm. in diameter. The carapace is flat dorsally, sub-circular in shape with an advanced, more truncate front than is present in the later females. The posterior margin is straight and the widest part of the carapace about $\frac{4}{7}$ the distance from the anterior end. The eyes are well developed. The front is about $\frac{2}{7}$ as wide as the carapace. The lateral margin of the carapace is thin and rather sharply bent from the dorsal side. Antennules are large and antennae small. The epistome is well defined. The buccal cavity is crescentic, arched and very broad from side to side but very short fore and aft. The external maxillipeds completely close the cavity and consist chiefly of the merus fused with the ischium and with the flagellum attached to the inner end. Carpus of the first segment of the palp or outer maxilliped, short, oblong; propodus more elongate, rounded; dactylus inserted behind middle of the propodus, minute and slender. Chelipeds (Fig. 5) [3] are stout, merus and carpus not slender as in the later females. Propodus slightly flattened inside, swollen outside and strongly widened from proximal toward distal end, narrowing again so that width at base of dactyli is considerably less than the greatest width of propodus. Both margins of propodus are convex. Fingers are stout, especially the immovable one with the tips hooked past each other when closed. The movable finger has a small tooth which fits between two protuberances on the immovable finger. Stiff hairs project from the gripping surfaces of both fingers.

The pereiopods are markedly flattened with the propodi almost spatulate, the third leg being slightly longer and stouter (Figs. 6–9). The propodi are widest about centrally, being half as wide as long and over twice as wide as thick (0.42×0.18 mm. in one case). The posterior border of the propodi of the walking legs is

[3] Although Figures 5–9, inclusive, were drawn specifically from a first stage female crab, the respective appendages of the same stage male are indistinguishable.

Plate II

Ventral view of chelipeds and pereiopods of Stage I and II female *Pinnotheres ostreum*. × 54

Figure 5. Cheliped of Stage I female.
Figures 6–9. Pereiopods of Stage I female.
Figure 10. Cheliped of Stage II female.
Figures 11–14. Pereiopods of Stage II female.

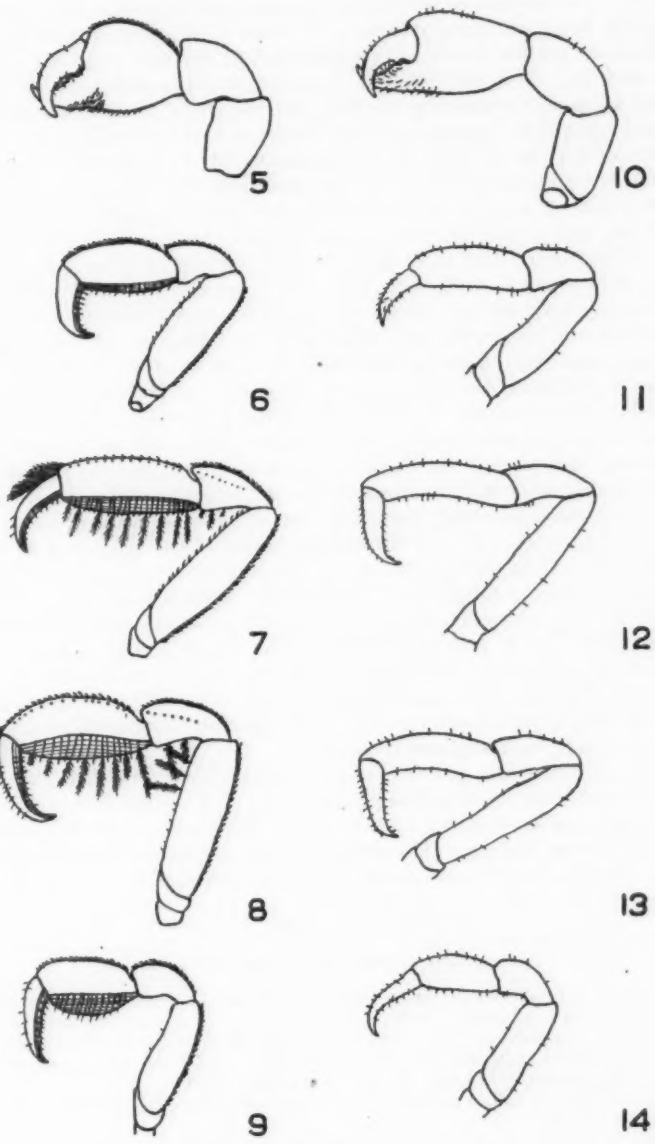

PLATE II

thickened. The amount of thickening is progressively greater from first to fourth walking leg, being not quite half the total width of the propodus of the fourth leg. The thickened portion seems to be constituted of the same material as the rest of the exoskeleton. Not only does it show longitudinal striations as though produced by successive depositions of chitin but the whole thickness is pierced by the hairs which protrude from the posterior border of each of the propodi. The dactyli of the same legs also show a similar thickening of the posterior which is likewise pierced by the protruding hairs. Unlike the female, the dactylus of the third leg of the male is longest but it is not markedly longer than that of the second which is almost as long and a trifle more curved at the tip. Also unlike the adult female, the dactyli of the second and third legs are only slightly more or less than half the length of the propodus of the same leg. There are two rows of long plumose hairs on the second and third legs. One row extends postero-dorsally from the anterior edge of the dorsal surface in a line running from midway on the carpus to the distal end of the propodus. Some of the hairs are over 0.6–0.9 mm. long. The other row extends backward from the posterior border of the flattened leg also extending from middle of the carpus to distal end of the propodus. These hairs are only about half as long as those on the anterior edge. The other joints of these legs and the other legs only possess short hairs or setae which are stout and plumose and are especially abundant on the anterior edges of the propodus, carpus and merus. We consider that these flattened, hairy legs are adaptations for free-living existence. Indeed, the first stage crabs are fair swimmers and reach the oysters by this means. The sides of the abdomen are almost straight and become narrower from the third to the seventh segment (Fig. 16). The terminal segment is arcuate. The abdomen fits neatly into the grooved sternum. A locking apparatus, somewhat like that described by Atkins for *P. pisum*, is present in the male *P. ostreum* (Figs. 15 and 16). It consists of a pair of almost conical chitinous knobs (on the fifth thoracic segment) along the side of the grooved area where the abdomen fits and a pair of larger blade-like protuberances on the sixth segment. Both sets of knobs project slightly anteriorly, ventrally, and medially.

Instead of fitting into grooves on the abdomen there are shelf-like projections on the fifth and sixth abdominal segments. The shelf on the fifth segment is much shallower and smaller than that on the sixth. The knobs on the thorax are hooked under these shelves making a close-fitting efficient locking mechanism that can easily be undone by the crab through extension of the abdomen and lifting but which is

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PLATE III

Some details of structure of *Pinnotheres ostreum*

FIGURE 15. Ventral view of the sternum of a male crab (Stage I) showing the chitinous knobs of the locking mechanism. × 20.

FIGURE 16. The abdomen of a Stage I male crab showing the copulatory pleopods. The first appendage on the right side was removed to better expose the smaller second appendage. Figure also shows the shelves of the locking mechanism. × 20.

FIGURES 17 AND 18. First and second pleopods, respectively, from the left side of a Stage II female crab. × 166.

FIGURES 19 AND 20. First and second pleopods, respectively, from the left side of a Stage III female crab. × 43.

FIGURES 21 AND 22. First and second pleopods, respectively, from the left side of a Stage IV female crab. × 22.

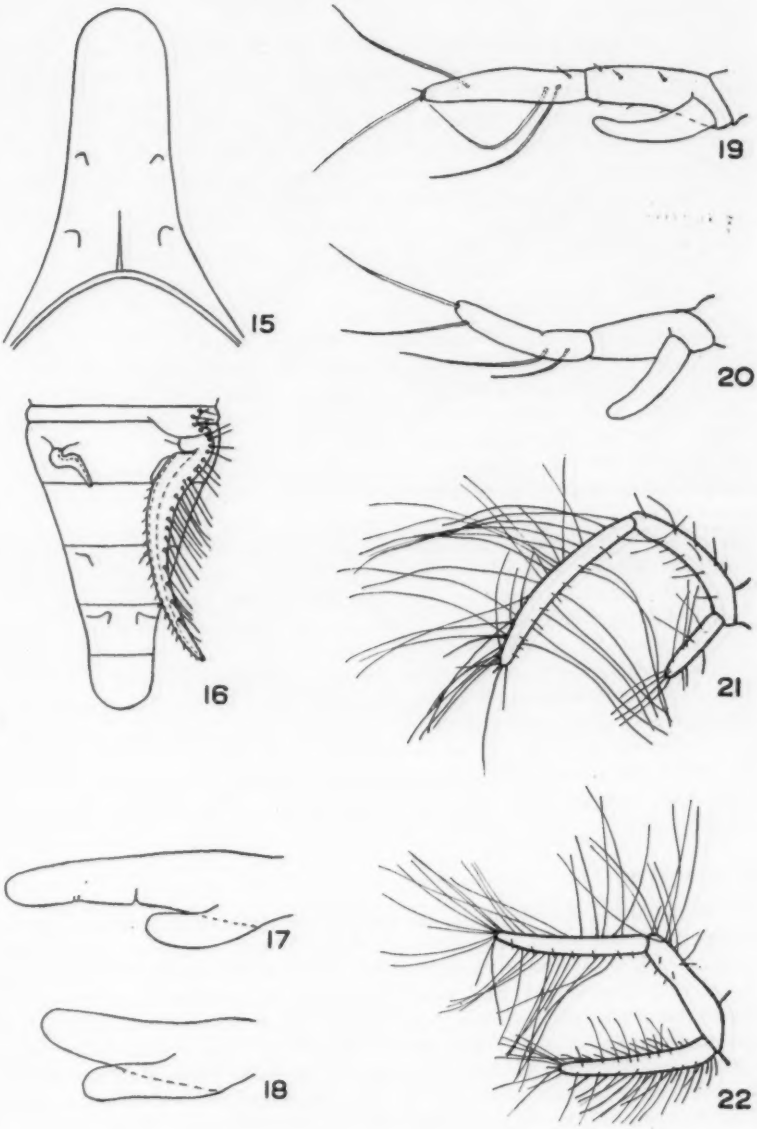

PLATE III

difficult to dislodge in the living animal where the stronger the flexing strain the more tightly the knobs lock.

The only thoracic somite dividing suture on the sternum is seen extending mediad from behind the coxa of the cheliped to a point a little more than midway to the groove where the abdomen lies. This is between the fourth and fifth thoracic segments. The copulatory organs of the male are large, like those in *P. pisum,* the first is blade-like and hairy and the second is rod-like (Fig. 16) and almost hairless. The tube runs along the medial side of the first appendage and the second appendage fits into a groove running across the under surface of the first appendage near the base of the terminal segment. The first appendage is more slender and scimitar-like than the same appendage on *P. pisum.*

First stage female

This is the female stage which invades the oyster (Figs. 1 and 3). It is superficially indistinguishable from the male although it is usually slightly smaller on the average (1.4–2.4 mm.). The mean of 187 measurements of width was 1.83 ± 0.14 mm. Not until careful examination is made of the abdomen and the sternal groove into which it fits can differentiation be made.

For example, there is only one pair of chitinous knobs (corresponding to the anterior pair of the male) and consequently only the terminal or corresponding pair of shelves on the abdomen (on the sixth segment). The genital aperture is small and located just posterior and medial to the base of the chitinous knob on each side. The relationship is thus almost precisely like that in *P. pisum.* The abdomen in the female has slightly straighter sides with the terminal segment having a lower arch. It lacks one pair of locking shelves and the segmentation between 3 and 4 and between 4 and 5 is much less distinct than in the male. Furthermore, the first segment is widest unlike the male where the third segment is widest. Such differences are very minor and usually cannot be determined *in situ* but only by removing the abdomen for comparison. Then, however, these differences are quite constant. Finally, the abdomen of the female bears four pairs of minute appendages, the first one of which is biramous, the second has a bilobed tip, presaging its eventual biramous state, and the other two are simple knobs. The walking legs are like those of the male.

Second stage female

This stage hardly exceeds in size the range of the first stage female. The carapace width of 67 specimens ranged from 0.9 to 3.1 mm. (mean 2.1 ± 0.23 mm.). The moult which occurs involves more differentiation than growth. The carapace is now smooth, shining, thin and membranaceous and, unlike the previous stage, yielding to the touch. It is convex from before backward and the lateral margins are thick and bluntly rounded. The front is still about $\frac{2}{7}$ the width of the carapace but now it is less truncate and does not project quite so far forward (Figs. 2 and 4).

The chelae are slightly more slender with the propodus widest more distally, nearly at the base of the fingers (Fig. 10). The pereiopods (Figs. 11–14) are slender and subcylindrical with delicate hairs on the last three segments. No such plumose hairs are present as described on the Stage I crabs. Propodus is not flattened on pereiopods as in Stage I nor does it broaden out very much, being only

PLATE IV

Photomicrographs of *Pinnotheres ostreum* and gill-eroded oysters. I wish to express my appreciation to Dr. T. C. Nelson for these. He not only took the photographs but found the particular specimens displayed. All × 3.

FIGURE 23. View of large-crab type gill erosions in an oyster. Note the presence of both a large Stage V female crab and a small Stage I male crab. The demibranchs of the oyster are shorter than normal and the ventral mantle cavity much enlarged.

FIGURE 24. Small-crab type erosions of gills. Note the sharply-delimited, punched-out area involving three demibranchs.

slightly wider than the merus of the same leg. The third leg again is largest and the dactyli of second and third legs nearly equal in length.

The abdomen is approximately the same shape as in the first stage. The ratio of carapace width to abdomen width is 2.46 and of abdomen length to abdomen width is 1.36. The first is still the widest segment. The terminal segment is only slightly wider (1½ times) than long in this stage. The sternum is still deeply grooved to hold the abdomen flush. Chitinous knobs are still to be seen on the thorax but the mechanism is now weakened by the delicate nature of exoskeleton.

This is obvious the first time one attempts to lift the abdomen. In Stage I males and females this is accomplished with difficulty and often the abdomen is injured in the process. In this and subsequent stages lifting of the abdomen is easy. Here is an adaptation to the sheltered life within the mantle cavity of the oyster which free-living first stages do not show. The legs also, no longer needed for swimming, have become slender and relatively smaller with each stage of development.

The abdominal appendages are but little advanced over those seen in the first stage female though in some instances they seem farther along than others. This and subsequent stages, while rather clearly defined, do vary quite broadly within the stage. In some there seems to be little change over the appendages of the first stage female, in others the biramous character of the first two pairs is more clearly marked. The appendages are still hairless, small and relatively undifferentiated (Figs. 17 and 18). There are many chromatophores scattered over carapace, sternum and abdomen. The legs have few of these black chromatophores although the chelae are well supplied. Consequently, the crab in this stage, against a light background, appears mottled grey with translucent appendages. Against a dark background, the appendages show up better and appear opaque. The later stages, especially the third stage female, are quite similar in color but cream-colored specimens are seen in increasing abundance. This is due chiefly to the decrease in numbers of these chromatophores and dispersion of pigment within them, and an increasing opacity of the organs within the crab. The adult female is always cream-colored except for the reddish outline of the mature gonad, the anterior parts of which are seen through the carapace from above and the posterior parts through the abdomen from below.

Third stage female

The chief differences are those of size, shape, and relative size of abdomen and character of the abdominal appendages. The width is from 2.6–4.4 mm. (mean of 31 specimens was 3.17 ± 0.40 mm.). It was found less frequently than first or second stage crabs in 1941 but has been relatively more abundant in 1942. The second and third walking legs are nearly equal in length with the dactyli also almost the same length. The average ratio of carapace width to abdomen width in eleven specimens was 2.0 and that of abdomen length to abdomen width was approximately 1.1. Thus the width of the abdomen has relatively increased slightly over both the length of the abdomen and the width of the carapace. In most of the specimens the third or fourth segment was widest. The terminal segment of the abdomen now has a flatter arch, usually being over twice as wide as long. The abdominal appendages, especially the first two pairs, are clearly segmented and possess a few distinctly plumose hairs (Figs. 19 and 20). The edges of the abdomen now extend beyond the broadened and shallower depression in the sternum which in earlier stages wholly contained the abdomen. There are hairs on the lateral edge of the abdomen in this stage. These hairs are more abundant on the more proximal segments and are quite variable in number. They are stout, plumose and rarely exceed 0.15 mm. in length.

Fourth stage female

Specimens on hand range in size from 3.6–8.9 mm. The abdomen is much broader now and just reaches the coxa of the legs in the majority of specimens.

It extends forward almost to the posterior edge of the mouth. The sternal depression is much broader in this stage though the abdomen overlaps it considerably. The third or fourth abdominal segment is widest and the abdomen wider than long even when pressed out flat. The carapace-abdomen width ratio has also further decreased and is now nearly equal to 1.0. The terminal abdominal segment has broadened to such an extent that it is now three to five times as wide as it is long. The abdominal legs are almost fully developed and well supplied with hairs (Figs. 21 and 22). The hairs along the lateral edge of the abdomen are more numerous and large and are present on all segments as a uniform fringe in the fourth stage crab.

The orbit has hardly increased in size and the diameter of the eye is practically the same. Thus the relative prominence of the eyes has decreased markedly. The carapace is now much more convex so that the front appears to be ventral in position. The abdomen is very convex externally and cannot be flattened without tearing the exoskeleton. Ventrally the hollowed abdomen is suited for carrying the masses of ova.

Fifth stage female

This is the stage commonly seen. The prominence of the reddish gonads identifies it in most cases though some fifth stage females are found with immature gonads. A few apparently fourth stage crabs show mature gonads, however. The features which differentiate the fourth and fifth stage females are rather slight. The abdomen is now wider than the carapace, extending to the basis of the walking legs in many cases. The terminal segment is more than five times as wide as long. The abdomen projects forward to a point anterior to the mouth which it covers except when feeding. For this act it rolls inward the terminal segments exposing the mouth parts as described for *P. pisum* by Orton. The size ranges from 6.0–14.9 mm. (34 specimens measured). It is possible that adult size is determined in part, as Atkins found for *P. pisum,* by the size of the host invaded. Even here, however, the range is great. Thus, a fifth stage crab 9.0 mm. wide was found in an oyster only 29 mm. long. Its abdomen was 12 mm. wide and extended beyond the coxopodites. No studies relating host size with crab size have been made but such a factor would account partly for the overlapping sizes of the various stages of the female crab. Another factor would be subsequent moulting after the fifth stage is reached. This is known to happen and several fifth stage females have moulted in the laboratory. Since only one moult of an earlier stage has been obtained which resulted in the production of a fourth stage female from a third stage crab, it is not known whether all earlier moults involve metamorphosis or whether growth moults can occur between stages. Biological variability and amount of available food are probably additional factors in size variations.

The walking legs of the adult female are different in their proportions from the earlier stages for the second leg is distinctly longer than the others and the dactylus of the second leg is more than two-thirds as long as the propodus. In our specimens, however, it does not seem to be regularly seven-eighths as long as Rathbun described it.

Two ovigerous females are present in our collection, the first measures 14.0 mm. across the carapace but the other only 8.9 mm. This is a further indication of the variability existing in the stages of the crab.

Second stage male

The presence of a number of specimens similar in external appearance to second or third stage females (body shape, exoskeleton yielding to the touch, slender, translucent legs, and grey or cream in color) has been noted. Thirteen measured specimens averaged 3.1 mm., ranging from 2.0–4.8 mm. Shape of abdomen and presence of typical male pleopods clearly identify these crabs as males. The pliable abdomen, however, only has one pair of the shelves of the locking mechanism though the sternum still shows both pairs of chitinous knobs. Like the females (except those of Stage I) the exoskeleton is delicate in these soft-bodied males and the abdomen can be easily lifted from the sternum even in living crabs. Since these crabs have been found in appreciable numbers it is quite possible that they may serve as partners in copulation with larger females of later stages though no evidence is available for or against this possibility. The size range would indicate that at least one, and probably two, moults can occur beyond the typical hard-shelled first stage males. It is also possible that these atypical males are the result of some sort of parasitism as Mercier and Poisson (1929) have reported for *P. pisum*. They found that males parasitized by the entoniscid, *Pinnotherion vermiforme*, resembled immature females in increased size and decreased firmness of the carapace and in the diminished number of hairs and greater slenderness of the legs. Parasitized female *P. pisum* were not perceptibly modified in these characteristics.

Presence of Pinnotheres in Oysters

All the stages of the crab described in the preceding pages were found in oysters from Delaware Bay in both 1941 and 1942. Although multiple infestations occurred involving various combinations of the crab stages, no case has been found containing more than a single specimen of either the third, fourth, or fifth stage crabs. First stage males and females were in great abundance in 1941 but were much less frequently seen in 1942 either per oyster opened or proportionate to the other crab stages. This is indicated in Table I where the same grounds of oysters were sampled in the two years. The only differences were that the oysters in each case were a year older in 1942 and that a twenty-five to thirty-three per cent mortality had occurred on each ground during the intervening winter.

Table I

Distribution of numbers and stages of P. ostreum *in oysters from two grounds sampled in consecutive years*

Ground	Year	Mean no. of crabs per oyster*	Max. no. of crabs per oyster	Oysters with crabs	Stage I crabs	Stage V crabs
A	1941	6.0	18	80%	91.4%	1.2%
	1942	1.1	3	73%	41.7%	33.3%
B	1941	4.2	19	80%	92.0%	1.6%
	1942	1.1	5	55%	50.0%	30.0%

* Based on total number of oysters in the sample.

We offer the data in this table also as additional evidence in favor of Atkins' belief (although she was working with *P. pisum*) that growth to maturity takes place within a year's time. The marked increase in both absolute and relative numbers of Stage V crabs in relation to a marked decrease in total crab population is more than suggestive.

The greatest number of crabs in a single live oyster was seen in 1941 when 262 crabs were found in an oyster measuring 85 × 46 mm. This oyster was in a sample dredged from Ground C already showing a high oyster mortality. Of the other live oysters in this sample, 54.6 per cent had more than 10 crabs present within the valves. Other high counts were 32, 63, 65, 81, 112, 134, 154, and 165 crabs per oyster. Multiple infestations have occurred even in small oysters, one dredged from Ground C, measuring only 32.4 × 14.7 mm., containing eighteen first stage crabs.

Besides oysters containing only a single specimen of any crab stage, almost all conceivable combinations of crabs have been found among the multiply-infested oysters except those involving the larger stages.

The only other cases of multiple infestations with crabs of the genus *Pinnotheres* were reported by Ohshima (1927). As many as seven male *P. latissimus* were found within the shells of a single specimen of *Paphia.*

The crabs when present in small numbers are usually found in the mantle cavity but in the heavier infestations crabs are found throughout the water conduction system of the oyster. An extreme case is that of the multiply-infested oyster dredged in September, 1942. The mature fifth stage female crab and five first stage crabs were found on the oyster's gills, three other first stage crabs were found in the cloacal chamber and one each in the promyal and the right and left suprabranchial chambers. In one interesting case the oyster when opened disclosed a Stage V female on the gills and a Stage II female in the promyal chamber. In another case three Stage I females were found one each in the promyal and left suprabranchial chambers and one at the exit of the promyal chamber.

When only a single crab is present it is usually found on the gills (in the mantle cavity) and females of Stages III, IV, and V have been found only in this area. We may state, therefore, that this is the normal position and that aberrant positions increase with the number of the invading crabs.

Orton (1921) reported a similar state of affairs with reference to *P. pisum* in *Mytilus edulis.* Observing the feeding of these crabs through windows in the mussel's shell he noted that the larger crabs sit on the middle of the mussel with a pair of gills on each side. The smaller crabs, he reported, may be found anywhere in the mussel but generally on the gill.

In Delaware Bay, oyster crabs are found in oysters from every area sampled to date. Since this included oysters well up the bay in regions of relatively less saline waters we may state that *Pinnotheres ostreum* is able to survive wherever the oyster can. Such a statement needs some qualification, however. Invasion of oysters by a new brood of young crabs probably occurs in late summer and autumn when the haligraphic picture of the bay waters (based on extreme data for more than seven years) is almost always at or above the mean annual level of salinities in the bay (Stauber, 1943). The meager data at hand indicate that if markedly low salinities prevailed at this critical time survival of the crabs would probably be greatly lessened. Crab-infested oysters subjected to adverse environmental conditions throw

considerable light on this problem. Death of first stage crabs within the oyster has occurred as a result of over-winter storage of oysters in pits in the ground and in a home refrigerator and as a result of the subjection of oysters to periods of low salinities (spring of 1941 and late autumn of 1942 on the beds far up the bay). It is especially notable that the later stages of the female crabs were able to survive these same adverse conditions. The latter fact is clear evidence that the adaptation of the crab to life within the oyster, as is indicated by the morphological changes of the female crab, involves physiological changes as well. Since the adverse situations described above all involve persistent closure of the oyster the female crab acquires the ability (which the oyster is well known to possess) to survive periods when cessation of the food supply and interruption of a steady stream of oxygenated water for respiration occur.

Invasions of oysters by *Pinnotheres* most probably occur in the late summer and autumn. This statement is based on several types of observation. The few ovigerous females seen have been encountered only during this period. Besides, crab zooeae of various species are found during these seasons. Furthermore, the 1941 mortalities began to be noticed in September of that year. Finally, infestation of oyster spat only a few months old was observed several times. Such invasion could only have occurred in the late summer or early autumn.

The heavy crab infestations of oysters reported in Table I were extreme values. In most areas sampled fewer than twenty-five per cent of the oysters opened contained crabs of any size, Stage V females being in the great majority. In such samples multiple infestations were very unusual. This is as valid for 1941 when the general incidence was much higher as it is for 1942.

Oysters of all sizes have been found infested with crabs. All stages of crabs have been found present though no later stages of females have been seen in oysters less than one year old for reasons which are obvious. The smallest infested oyster was one less than four months old from the egg and only 20 × 16 mm. in size. A second stage female crab measuring only 0.9 mm. wide was found in the mantle cavity. The left mantle was perforated and the inner right demibranch slightly eroded. These observations indicate that growth and differentiation of the crab and perceptible damage to the oyster can occur within a relatively short period of time.

The smallest oyster yet opened which has contained a mature fifth stage female was an oyster probably less than two years old and measuring only 34 mm. long. The crab had a carapace 9 mm. wide and large-crab type gill erosions were present.

Mode of Feeding

Orton's description of the feeding of *P. pisum* in *Mytilus edulis* is remarkably similar to the method of feeding of *P. ostreum* in the oyster. The larger crabs are stationed with ventral side approximated toward the inner aspect of one mantle and facing away from the oyster's gills toward the ventral margin of the shell or into the incurrent stream of water. Usually two of the oyster demibranchs project over and above the carapace of the crab and not in regular contact with the crab. The other two demibranchs are close to and at times in contact with the posterior part of the ventral surface of the crab.

The oyster feeds by straining sediment and plankton (chiefly diatoms and dino-

flagellates) from the water.[4] A portion of these strained particles mixed with mucus is passed anteriorly in trails along the marginal furrow of each demibranch. The palps of the oyster constitute a further sorting apparatus and some of the strained material is rejected by the palps. This rejected material is passed posteriorly and ventrally along a ciliated path on the inner aspect of the mantle to a point near the ventral edge of the shell from which it is ejected by the blowing force of the sudden closure of the oyster's valves. If the oyster crab confined its feeding activity to the constant stream of rejected material on its way from the palps it might be of some value to the oyster and could possibly be called a symbiont or at least a commensal. However, in our experience the rejected masses are too bulky to easily fit into the crab mouth and most of this material is eventually thrown off by the crab after attempting to break it up into suitable particles by the action of the mouth parts. By reaching beneath its abdomen with its claws and also by disengaging the tangled mucus-food masses caught in its legs, it obtains newly-formed, delicate, strings of food much more capable of being handled by the mouth parts and ingested. I have seen such strings completely and quickly engulfed. The crab often combs the other legs with the chelipeds to free them from entangled food masses. The mucous masses are then passed to the mouth where the mouth parts in turn gather and press the strings of food into the mouth. The fourth or last pair of walking legs are frequently worked up over the carapace of the crab to scrape forward within the easy reach of the chelipeds any food strings which may fall from the other demibranchs or even to disengage them from the marginal furrow. The large crabs range in the mantle cavity from the place where palps and gills meet to a point ventral to the adductor muscle. Under favorable conditions they are seen to be very active, almost constantly moving legs or chelipeds.

The process of feeding, however, is wasteful and much of the material brought to the mouth is handled several times before it is ingested with appreciable amounts thrown off by the mouth parts. In one case a large crab was straddling the stream of rejected mucus-food passing to the edge of the mantle from the palps yet in more than fifteen minutes' steady observation it failed even to attempt to pick it up. In the meantime the combings of the walking legs were being vigorously prosecuted.

Rathbun remarks that the food of the oyster crab is composed of the same organisms which constitute the food of the oyster plus small crustaceans not normally the food of the oyster. The complicated feeding mechanism of the oyster is chiefly one of straining particles from the water and then sorting out and discarding the larger of these particles. Much of the food passing along the marginal furrow is destined to be discarded because of particle size. It is not surprising, then, that small crustaceans (Rathbun) are included in the crab's diet in addition to the entangled smaller organisms eaten by the oyster.

Observations on the feeding of the smaller stages of *P. ostreum* (especially first and second stages) throw further light on the mechanism producing the gill damage to be described later. In maintaining its position on the demibranchs the pointed dactyli of the walking legs are regularly seen to pierce the gill tissue. In order to maintain its position several nearby plicae may be stretched out of position by the pull of the inserted dactylus. When the leg is moved the area pierced stands out as

[4] This description is based chiefly on the findings of Yonge (1926), Elsey (1935) and especially Nelson (1938 and personal communications).

an opaque spot on the demibranch. It appears like a condensation of tissue either by compression of surrounding parts or by invasion of leucocytes. This area is visible to the unaided eye for several hours. Some of the walking legs have been observed to scrape the gill in an attempt to engage mucous strings of food moving on the filaments (especially the principal filaments in the folds between the plicae). This scraping action may be very vigorous and may be likened to the child's use of its fingers to clean the frosting from the egg beater. Occasionally the dactylus of the scraping leg catches in a portion of the gill (ostium). The crab strains and the gill tissue stretches. When the pull becomes hard enough the leg finally completes its motion (due to the local tearing of the gill) and the affected portion of the demibranch springs back into position. The leg and cheliped would then be approximated and the combing would proceed even when no food material had been obtained by the active leg. (Since many of these observations were made in very clear water there was little material to be filtered by the oyster.) Small crabs have also actually been seen to grasp the delicate gill tissue with the cheliped and put tension on it, pulling it out of normal alignment. When released the gill was seen to be definitely injured.

In all stages of the crabs the activity was seen not to consist so much of movement from place to place but chiefly movements of legs and chelipeds related to the act of feeding.

Injury to the Oyster Gill and Gill Regeneration

Although no extensive erosions of gills were produced by crabs in the laboratory, nevertheless, the observations recorded and the activities and injuries discussed above are sufficient to account for the extensive gill damage regularly seen in crab-infested oysters.

Two general types of gill damage were noted associated with large and small crabs respectively. The large-crab type erosion (Fig. 23) shows extensive shortening in the height of one or more demibranchs over an area reaching from the anterior end of the gills where they meet the palps to a point usually ventral to the adductor muscle. The affected demibranchs may be even less than half as high as would be expected normally or as the unaffected demibranchs of the same oyster. The outer demibranchs are normally shorter than the inner ones but cases where both inners and one outer are eroded have been seen. Such specimens show the uneroded outer demibranch to be the highest but only in the region where the other gills are eroded. The edges of the eroded demibranchs are usually slightly ragged, markedly thickened, and considerably more opaque than the edges of normal oyster gills. As a result of the gill erosions as well as the more rapid growth of the mantle and shell in a ventral direction along with the growth of the crab, an enlarged mantle cavity is produced permitting ample room for the movement of the contained crab. One oyster examined demonstrated the extreme condition which could develop as a result of the presence and activity of a large crab. The oyster was 7.0 cm. long from hinge to bill and 4.8 cm. wide just anterior to the adductor muscle. The periphery of the mantle extended to the edge of the shell in all directions. The distal margin of the demibranchs, however, did not reach the ventral shell margin by 2.1 cm. at the widest part of the oyster. The left demibranchs were only 0.8 cm. high and the eroded right demibranchs only half this size. The fifth

stage female crab was 13.0 mm. wide but with outstretched legs and chelae she extended over an area 4 by 2 cm. At first glance after turning back the mantle the body of the oyster appeared to occupy only the dorsal half of the cavity formed by the two shells.

The position of the crab within the oyster was studied in relation to the orientation of the oyster on the bottom. It was found that the crab always faced away from the gill with the normal gills over the crab's carapace (Fig. 23) even when the oyster's position on the bottom made it certain that the crab had remained consistently upside down with relation to the earth's gravitational field. In the laboratory also, there were numerous cases where the crabs remained for days in an upside down position on the oyster's gills. It is this persistent maintenance of position associated with the feeding activities of the crabs which produces the type of erosions seen.

The small-crab type erosion is quite different in appearance and consists of a more local, sharply delimited erosion of one or more demibranchs (Fig. 24). Any single or contiguous demibranchs may be eroded or even all four. The earliest cases show a ragged distal edge, but further attack gradually hollows out a space in which the crab is found. The picture reminds one of a portion cut away or punched out of the edge of the gill although in two cases one of the eroded demibranchs was perforated. The margins of the damaged area are usually thickened by what appears to be accumulations of leucocytes and probably regeneration of damaged tissue. Small-crab type erosions are usually seen at the anterior ends of the demibranchs though, occasionally, typical erosions are observed at any point along the gill. The demibranchs are often eroded more than half way to the base. Very rarely in multiple infestations two areas of damaged gill are seen. Except for one case of eroded palps and several cases with a perforated or damaged mantle area in close approximation to typically eroded gills no other sites of injury to the oyster have been found. In a few cases the mantle instead of being actually perforated was blown up into a "blood blister" or a "pus pocket" as a result of the irritating activity of the crab. The localization of injury is almost conclusive proof that gill destruction is produced by the impingement of the crab's method of feeding on the oyster's food-sorting equipment. It is most likely that the initial break in the marginal furrow by causing food trails to end at the break results in intensified activity by the crab at this spot. Such gill damage is not necessary to but incidental to the mode of feeding of *Pinnotheres*. In the case of the first stage crabs, however, the exoskeleton is hard and unyielding. Certainly this contributes to the production of the early lesions though extension to the large-crab type must occur in later stages of development. Since the adaptation of the crab to the oyster has not yet reached a plane where neither suffers injury the oyster-crab, *Pinnotheres ostreum*, must be considered parasitic on the oyster. It is conceivable that further evolution by shortening the feeding time of the first stage crabs, further decreasing the strength of the exoskeleton, increasing the skill with which the crab obtains the mucus-food strings or confining its feeding to the palp-rejected masses, might result in a relationship less harmful to the oyster. The crab would then be a more efficient parasite in Swellengrebel's (1940) sense of the word.

In small-crab type erosions the gill damage frequently extends down to the base of the gill such that the suprabranchial chamber is directly visible through the most proximal portions of the water tubes. Previous types of damage described involve

chiefly a decrease in total gill surface available for feeding. The exposure of the water tubes illustrates another type of malfunction of the oyster's feeding mechanism which has been studied in more detail in the laboratory.

When the oyster's gills are experimentally damaged it can be shown that such injuries produce leaks in the water conduction system of the oyster. Since such a leak backward into the mantle cavity means that water already strained must be rehandled several times a decrease in gill efficiency results the degree of which depends on the extent of the leakage. In a typical experiment a dissecting needle was pushed into the inner left demibranch of a small oyster (No. 1) on the half shell (opened six days before) and forced distad tearing a single plica for a distance of approximately 1.8 mm. from the marginal furrow. When the surrounding plicae were tested with ink suspension [5] some of the latter was seen to escape from the torn plica. A portion of the water being strained by six adjacent plicae was observed to be leaking back into the mantle cavity.

When more extensive V-shaped areas were cut out of a demibranch by scissors the leakage was much greater in volume and water strained from a much larger area was involved. A market-size oyster (No. 2) opened and studied on the half shell will serve as a typical example. Such preparations live as long as six weeks in aquaria, especially if water temperatures are generally below 20° C. A wedge of the outer left demibranch was cut out which measured about 1 cm. across the widest part (along the marginal furrow). At the apex the cut penetrated to a point slightly more than halfway to the gill axis or base. The excision was located just posterior to the anterior third of the demibranch. When ink suspension was placed on this demibranch anterior to the cut practically none escaped in the normal fashion from the cloacal chamber but streamed forcefully out of the openings of the severed water tubes. In consequence, the water strained by more than a third of one demibranch had to be rehandled. Ink placed on the inner left demibranch at the level of the cut on the outer left demibranch also escaped in large part from the cut water tubes of the outer left demibranch. Such a loss of efficiency of parts of two gill leaves must reduce the amount of food an oyster can obtain.

As a result of these findings newly-opened, crab-infested, gill-eroded oysters were studied to see whether such leakages occurred naturally. For reasons to be discussed later; namely, the rapid rate of healing and regeneration of the oyster, good examples were hard to find. One notable case, however, examined in January, clearly demonstrated the existence of such injuries. Water tubes of the two inner demibranchs were exposed in the central portion of a rather extensive small-crab type erosion. When tested on the half shell with ink, both affected demibranchs showed significant leaks. Not only the usual loss from ink placed anteriorly to the lesion but even some placed posteriorly escaped from the damaged area. The latter indicates that some water was moving *anteriorly* in the suprabranchial chamber above the inner right demibranch for a short distance.

[5] The ink suspension is made by diluting Higgins' Eternal Black Carbon ink with bay water. A dilution of about 1–10 with some subsequent evaporation makes a suspension suitable for use. Placed on the gill of a normal oyster the particle sizes are generally so small that almost all the ink is swept through the ostia into the water tubes. In the intact oyster it appears as a cone-shaped black cloud issuing from either or both of the exhalent chambers on the dorsal side of the oyster.

In another experiment a small oyster on the half shell was placed in a dish with four second stage female crabs. Three days later parts of the demibranchs showed twisted and fused plicae (healed), broken plicae and irregularities of form of filaments not normally seen. One particular area showed extensive damage of nine plicae including destruction of the marginal furrow of eight of these.

To explain the fact that serious leaks are not often naturally found we studied the daily changes in experimentally produced gill lesions of oysters on the half shell. The lesions healed in such a remarkably short period of time that we can easily understand our previous difficulties.

In oyster No. 1, for example, twenty-four hours after the gill injury was produced marked healing had occurred. Three days later (water temperatures in intervening period below 10° C.) much of the damage had been repaired. Anatomically, as well as by ink test, the hole had been plugged by tissue though reorganization of filaments and the marginal furrow had not occurred.

Another small oyster (No. 3) healed much more rapidly. In this case ink escaped from three small experimentally injured areas when tested soon after injury. Less than 24 hours later the holes were sufficiently bridged over by local contractions and regeneration to make water tight plugs.

Another small oyster receiving wedge-shaped excisions of both left demibranchs healed over the exposed water tubes of the shallower one and more than half of the more seriously damaged one in less than a day. Total closure of the wound was accomplished by the fourth day.

Oyster No. 2 likewise healed over though here the excision was so broad and deep that a longer period was necessary for recovery. At the twenty-four interval little microscopically-visible healing could be seen but loss of ink occurred only when placed over a space extending twelve plicae anterior to the excised area. A large amount of ink placed on the inner left demibranch at the level of the injury still showed some loss from the exposed water tubes of the outer left gill leaf.

By the fourth day almost half the exposed openings were bridged over with tissue. On the sixth day ink placed more than two plicae anterior to the edge of the excision no longer escaped from the cut water tubes. When placed proximal to the cut, ink escaped from less than half the originally exposed water tubes and then only at the apex of the wedge where the most exposure occurred. Now, also, only a small amount of the ink placed on the other demibranch (inner left) was lost at the cut surface. By the tenth day microscopic examination and ink tests showed that the gap had been completely closed. Although the damage was not yet all repaired the most serious effects were circumvented. It is reasonable to suspect that the intact oyster under more favorable conditions could respond even more quickly than has been demonstrated in these experiments. Since the crab probably does not confine its activity to a single plica for any considerable length of time a reasonable explanation of the appearance of the gill erosions in recently-opened oysters is at hand.

Gill-damaged oysters lose their crabs at times or eject dead ones. Eventual reconstitution of essential gill structure may then take place though cicatrization and "scar" formation are noticeable. None of the interesting histological aspects of the healing process have yet been studied.

Other Effects of Invasion

Oysters exposed to prolonged storage in air or earth were found to vary in their keeping qualities. It was soon noted that most of the weaker oysters were crab-infested. Whether this was due to the poorer condition of the oyster, irritation by the crab or greater loss of shell liquor due to the enlarged mantle cavity is not known. From the commercial viewpoint, however, this is a serious matter. Much of these losses automatically are circumvented because the oysterman selects for market only his best oysters. It should be emphasized here that even in multiple infestations the presence of more than one gill erosion is extremely rare. An oyster containing fifty crabs is more likely to have no greater eroded area than an oyster containing only a single crab. It is our belief, therefore, that multiple infestations are the result not only of the presence of large numbers of invasive crabs in the water but also of the weakness produced by the activity of a primary crab invasion or any other condition producing weak oysters. The malfunction or even complete stoppage of the feeding mechanism under such conditions may account for the lack of further gill erosions. Such oysters were probably unable to survive the winter and contributed to the heavy mortalities which occurred in some areas after the winter of 1941–1942.

Invasion of Oysters by Crabs

Orton believes that the hard, flat shell of the invasive first stage crab (*P. pisum*) is of value not only in slipping within the valves of the mollusc but in avoiding crushing by the closing valves. We believe this to be largely true for *P. ostreum* and the oyster, though successful invasion does not always occur.

In one experiment an oyster dredged for market was placed in an aquarium with one hundred and fifty first stage *Pinnotheres*. The crabs were scattered over the dish at the start of the trial. Within a short time many were seen lined up around the oyster from hinge to bill mostly at the places where the two valves come together. Seven days after the start only twenty-five live and twenty *dead* crabs were found in the dish but none were now present on the oyster. The oyster was then opened. There were forty-nine live crabs in the left valve on opening, most of these were around the edges of the shell on the incurrent side where the oyster had apparently begun to wall off the crabs. Twenty-six more crabs were found on the ventral side of the oyster, mostly on the gills but *no gill erosions* were present. Sixteen crabs were on the dorsal side, three in the promyal chamber, two deep in the cloacal chamber, three deep in the left suprabranchial, three in the right suprabranchial chamber and two so deep in the water tubes of the anterior end of the inner right demibranch that the gill had to be dissected to free them. The oyster was 108 × 66 mm. in size and contained one hundred and four of the crabs. All but one of the original crabs were found. Two of the other trials with various oysters yielded invasions of seventy-seven of one hundred and eighty-one and forty-nine of one hundred and twenty-nine crabs.

In all trials a few dead crabs were seen and in one series of closely examined oysters exposed to only a few crabs the fate of several was followed. A typical case showed a crab attached to the oyster shell opposite the opening of the cloacal chamber. It had assumed this position within a half hour and almost appeared as

though its posterior end were caught within the oyster's shell. Seven hours later it had scarcely moved in position but one hour later it was found on the bottom of the dish on its back, dead, directly below the position it had been occupying on the shell of the oyster. It had apparently attempted entry and partially succeeded but was compressed until dead and then ejected. In another case a weak crab was removed from the bottom of a dish for examination. It had lost parts of two legs on one side. In still another instance two specimens which had effected entry, so far as could be detected, were later ejected from the oyster as dead crabs. These probably had effected almost but not complete entry.

Note on Collecting Specimens for Future Study

In commercial packing of oysters the oysters are drained—in part to insure accurate measurement of the volume of the oysters opened. This volume is the basis of paying the shucker or opener. The oysters are later washed and drained again before shipment. Catching the first drainings in pails and then placing the material collected in shallow pans or dishes of bay water allows the crabs, freed in the opening of the oysters, to be readily recognized and collected.[6] We have been successful in obtaining specimens of all stages of *P. ostreum* in this fashion especially from areas where crab density is very low.

Discussion

It is obvious from the data presented that *Pinnotheres ostreum* can no longer be considered other than a parasite of the oyster. That we witnessed a most unusual sequence of events in 1941 is without question for the dramatic appearance of an oyster containing one hundred or more crabs can hardly be overstated. The subsequent decrease of numbers in 1942 is another example of the population cycles so often seen in nature. We have also seen the abrupt rise and fall in numbers of starfish, *Stylochus ellipticus* and *Polydora ligni* (Nelson and Stauber, 1940), on the oyster grounds in Delaware Bay in recent years. The rise of each was associated with heavy, localized mortalities.

It is not surprising that the evidence gathered here was not previously noted. The small size of the first stage crabs and the need for careful opening and search of oysters in order to detect their presence are prime reasons. For example, we have repeatedly seen cases with gill erosions but could detect no crab on the first examination. Placing these oysters on the half shell in dishes of bay water for a few hours a subsequent examination would often reveal the presence of a crab. Furthermore, among oysters held out of water for a time we have found specimens of first stage *P. ostreum* dried fast to the edge of the oyster's shell as though they had attempted to leave the unfavorable environment and were trapped by lack of an ambient fluid medium in which to move. This being the case it would take such an unprecedented increase in density as occurred in 1941 to focus one's attention on the causal relationship between crab and oyster gill damage. The very fact that in over one hundred years only two specimens of male crabs were obtained by collectors is in itself suggestive. Nor can the fact that oystermen always select for quality of meats be overlooked. Only someone searching for and among poor oysters or investigating causes of mortality would be apt to find the crabs.

[6] I am indebted to Dr. T. C. Nelson for this fruitful suggestion.

The large crabs, however, have long been known and their very existence means that the earlier stages were also present though undetected. Dean's description of malformed or stunted palps with thickened outgrowths is clearly indicative that the parasitism has not been acquired recently. But the shortening of demibranchs on the affected side of an oyster containing a Stage V crab is not always readily distinguished especially since there are minor differences in the heights of the demibranchs normally. In a significant proportion of the cases also, since the erosions are present on only one or two of the demibranchs, the damaged area is shielded from cursory glance by the unaffected gills which cover them. This is even true of the small-crab type lesion in many cases.

It would be of great interest to learn whether these findings are duplicated at other points along the eastern seaboard where the presence of *Pinnotheres ostreum* within the valves of *Gryphaca virginica* has long been known to exist.

Summary

1. All the parasitic stages of *Pinnotheres ostreum* are described, many for the first time. Besides the invasive first stage male and female crabs, one other male stage and four other female stages are reported.

2. The first crab stage (male or female) is hard-shelled, relatively flat and hairy with a distinctive brown color and white markings.

3. Subsequent stages are quite different with membranaceous, yielding carapace, slender legs, and more rounded body form.

4. The later stages of the females are distinguished from one another by size, color, width and shape of abdomen with relation to the rest of the body and size and differentiation of the pleopods. Maturity is usually reached in the fifth stage female and ovigerous crabs have been seen.

5. All stages of crabs have been found in oysters either singly or in various combinations except that no more than one specimen of a Stage III, IV, or V female has ever been observed within a single oyster.

6. Multiple infestations have been observed which involve chiefly first stage crabs. One oyster contained 262 of these crabs.

7. The oyster crab feeds chiefly on the particulate food material strained from the water by the oyster's food sorting mechanism.

8. In so feeding the crab injures the demibranchs of the oyster causing erosions which interfere with normal feeding of the oyster. These erosions are sharply localized in oysters with first stage crabs.

9. Damage to the oyster gills involves a reduction in the amount of area available for food collecting and in the efficiency of both the straining and the collecting mechanisms. The more serious lesions may also show leakages of the water pumping or conduction system which is such an important feature of food sorting.

10. Rapid healing and regeneration of gill tissue almost keeps pace with destruction and probably saves many oysters from death.

11. The hard shelled, almost tick-like, invasive first stage crabs are less adapted to life within the oyster than the modified later stages. The latter are more resistant to their host's exposure to unfavorable environmental conditions such as storage out of water.

12. Experimental invasion of oysters by crabs has been accomplished but artificial duplication of typical, extensive gill lesions has not yet been obtained.

13. The unprecedented invasion of oysters in 1941 was followed by a lesser invasion in 1942 and probably will follow the usual course of population cycles in future years.

14. The parasitic nature of the crab's relations with the oyster is outlined and reasons suggested why this previously was not found out.

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